

The Regulation of Nitric Oxide by Intracellular Glutathione in Lung Epithelial Cells.

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ABSTRACT

Nitric oxide (NO[•]) is known to be present in increased amounts in the breath of acute asthmatics and evidence suggests that airway epithelial cells release NO[•], particularly in response to pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α). NO[•] is synthesised by the conversion of L-arginine to L-Citruline by the enzyme NO[•] synthase (NOS), which is present in a wide range of cell types and can be both inducible and constitutive. There is evidence that oxidative stress can induce NO[•] synthase (NOS) mRNA synthesis in airspace epithelial cells in vitro, through the activation of the transcription factor NF- κ B. It has been shown that NF- κ B activation is necessary for inducible NOS (iNOS) induction and NF- κ B is thought to be oxidant regulated, either directly by the oxidants themselves, or by the antioxidant state of the cell. Cytokines, such as TNF- α , which result in increased NO[•] release from lung epithelial cells may act on the mitochondria, increasing mitochondrial release of superoxide anions, which may, in turn, activate NF- κ B resulting in iNOS induction and NO[•] release. Glutathione (GSH) is a ubiquitous low weight molecular thiol, which is required for many cellular functions. Moreover, as a major antioxidant it has a role in maintaining the reduction/oxidation (redox) potential of the cell and is important in protecting the cells against oxidative damage, such as that induced by superoxide anions. There is evidence to suggest that the ratio of oxidised GSH (GSSG) to reduced GSH may be critical in oxidant induced NF- κ B activation. The level of GSSG is thought to be involved in the modification of proteins involved in the cascade leading to NF- κ B activation and in the degradation of I κ B, the inhibitory subunit of NF- κ B. It has also been shown that NF- κ B requires a reductive environment for DNA binding and GSH levels may therefore affect NF- κ B binding to DNA. Furthermore, induction of NO[•] by cytokines and oxidants is associated with a decrease in intracellular GSH in several cell types, and thus it is likely that an oxidant/antioxidant balance is important in the regulation of NF- κ B activation. The hypothesis of this thesis is that changes in intracellular GSH redox state effect iNOS mRNA, via NF- κ B activation, in response to both cytokines and oxidants. The data presented show that both cytokines and oxidants (H₂O₂) induce iNOS mRNA and NO[•] release in both the human Alveolar Type II epithelial cell line (A549) and the

human Bronchial Epithelial cell line (16HBE14o-) and this NO[•] release is concomitant with a decrease in GSH. The decrease in GSH is not, however, due to the production of NO[•] as shown by the use of NO inhibitors supporting a role for GSH in NO[•] induction. Decreasing intracellular GSH levels with BSO caused an increase in iNOS induction and NO[•] release suggesting that decreased GSH levels may have a role in iNOS induction. GSH levels were increased by 4 thiol compounds to determine if increased GSH levels could prevent or decrease iNOS induction. Increased GSH levels decreased H₂O₂ induction, but not cytomix induction of NF-κB activation and NO[•] release. This suggests that increasing oxidant stress directly, using an oxidant, results in NF-κB activation and iNOS induction, and this increase can be inhibited by increasing intracellular GSH levels. However increasing NO[•] release and NF-κB activation with a mixture of cytokines is not, however, decreased by increased GSH levels, suggesting an alternate pathway exists for NF-κB activation and iNOS induction by cytomix. In conclusion, this data supports the hypothesis that the redox status of the cell, particularly GSH is involved in the activation of NF-κB and induction of iNOS by oxidants. GSH does not, however, appear to play a critical role in activation of NF-κB and induction of iNOS by cytomix suggesting iNOS induction can be achieved by different signalling pathways. These data may be important in inflammatory disorders of the lung and may provide information on the uses of antioxidants as a means of inhibiting NO[•] during lung inflammation.

DECLARATION

The work presented in this thesis was carried out solely by the author, unless otherwise stated, under the supervision of Professor W. MacNee and Professor K. Donaldson, Wilkie Laboratory, University of Edinburgh.

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ABBREVIATIONS

16HBE14o ⁺ (16HBE)	Human bronchial epithelial cell line
A549	Human alveolar type II cell line
AA	Non-essential amino acid mixture
ABC	Avidin biotinylated complex
ALF	Airway lining fluid
APS	Ammonium persulphate
ARDS	Adult respiratory distress syndrome
BAL	Bronchoalveolar lavage
BCNU	Bis 2 chloroethyl nitrosourea
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
cDNA	Complementary DNA
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
cNOS	Constitutive nitric oxide synthase
CO ₂	Carbon dioxide
COPD	Chronic Obstructive Pulmonary Disease
CuZn SOD	Copper-zinc superoxide dismutase
DAB	Diaminobenzidine
DEM	Diethylmaleate
dH ₂ O	Distilled water
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
ELF	Epithelial lining fluid
EMSA	Electrophoretic mobility shift assay
Fe ²⁺	Ferrous iron
Fe ³⁺	Ferric iron
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase.
γ-GCS	Gamma glutamylcysteine synthetase
γ-GT	Gamma glutamyl transpeptidase,
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glutathione synthetase
GSH	Glutathione (reduced)
GSHMEE	Glutathione monoethyl ester
GSNO	Nitrosothiols
GSSG	Glutathione disulphide (oxidised)
H ₂ O ₂	Hydrogen peroxide
H ₃ PO ₄	Phosphoric acid
HOCl	Hypochlorous acid
IFN-γ	Interferon gamma

IκB	Inhibitory protein kappa B
IL1-β	Interleukin 1- β
iNOS	Inducible nitric oxide synthase
IRF-1	Interferon regulatory factor 1
KCl	Potassium chloride
L-G	L-glutamine
LDH	Lactate dehydrogenase
L-NMMA	N ^G -monomethyl-L-arginine
LPS	Lipopolysaccharide
MEM	Minimal essential medium
MgCl ₂	Magnesium chloride
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
NAC	N-acetylcysteine
NaCl	Sodium chloride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NAL	N-acetylcysteine L-lysinate
NF-κB	Nuclear factor kappa B
NPSH	Non-protein thiols
NO [·]	Nitric oxide
NO ₂	Nitrogen dioxide
N ₂ O ₃	Dinitrogen trioxide
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
O ₂	Oxygen
O ₂ ^{-·}	Superoxide anion
OH [·]	Hydroxyl radical
ONOO [·]	Peroxynitrite
ONOOH	Peroxynitrous acid
P	Penicillin
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMN	Polymorphonuclear monocyte
PMSF	Phenylmethanesulfonyl fluoride
RAM	Rabbit anti-mouse
ROOH	Organic hydroperoxides
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
S	Streptomycin
SF	Serum free
SOD	Superoxide dismutase
SSA	Sulphosalicylic acid
TBE	Thiobarbituric acid
TEA	Triethanolamine
TEMED	N',N',N',N', Tetramethylethylenediamine
TNF-α	Tumour necrosis factor-alpha

2-VP
XO

2-vinylpyridine
Xanthine oxidase

Table of Contents

Chapter 1 Introduction.....	4
1. THE LUNG AND ROS.....	5
1.1. STRUCTURE OF THE LUNGS.....	5
1.2 INFLAMMATORY CELLS.....	7
1.3 REACTIVE OXYGEN SPECIES (ROS).....	8
1.4 ROS AND OXIDATIVE DAMAGE.....	11
2 Antioxidants.....	13
2.1 THE PREVENTION AND COMPARTMENTALISATION OF FREE RADICALS.....	14
2.2 THE CONVERSION OF OXIDANTS TO LESS REACTIVE SPECIES.	14
2.3 REPAIR OF OXIDANT INDUCED INJURY.....	17
2.4 EXTRACELLULAR ANTIOXIDANT DEFENCES.	17
2.5 NO [•] AS AN ANTIOXIDANT.....	18
3 Inflammatory Disorders Involving ROS And Antioxidants.....	19
3.1 ASTHMA.....	19
3.2 ARDS.....	21
3.3 OTHER RESPIRATORY DISORDERS ASSOCIATED WITH INCREASED ROS.....	22
4 Nitric Oxide (NO[•])	24
4.1 BACKGROUND	24
4.2. METABOLISM/BIOCHEMISTRY	25
4.3 NO [•] AS A FREE RADICAL.....	28
4.4 NO [•] AS A MESSENGER AND TOXIN.	33
4.5 NO [•] IN LUNG DISORDERS	34
5 Glutathione (L-γ-glutamyl-L-Cysteinylglycine)	38
5.1 GSH METABOLISM.....	39
5.2 GSH AND THE LUNGS.....	42
6. Nuclear Factor–KappaB (NF-κB).....	43
6.1 STRUCTURE	44
6.2 THE INHIBITORY SUBUNIT IκB.....	45
6.3 ACTIVATION OF NF-κB	46
6.4 REDOX REGULATION OF NF-κB.....	46
7 Hypothesis.....	50
8 Aims.....	50
8.1 SPECIFIC OBJECTIVES:.....	50
Chapter 2 Materials and Methods.....	51
2.1 MATERIALS.....	51

2.2 METHODS.....	51
2.2.1 CELL CULTURE TECHNIQUES.....	51
2.2.2 EXPERIMENTAL TREATMENT OF CELLS	53
2.2.3 ASSAYS.....	54
2.2.3.1 PREPARATION OF CELLS PRIOR TO ASSAYS.....	54
2.2.3.2 CELL COUNTS AND CELL VIABILITY.....	55
2.2.3.3 NITRIC OXIDE (NO [•]) ASSAY.....	55
2.2.3.4 REDUCED GLUTATHIONE (GSH) ASSAY.....	56
2.2.3.5 OXIDISED GSH (GSSG).....	56
2.2.3.6 MODIFICATION OF TIETZE METHOD.....	57
2.2.3.7 TOTAL PROTEIN ASSAY.....	58
2.2.4 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION.....	59
2.2.4.1 RNA EXTRACTION.....	59
2.2.4.2 CDNA PREPARATION.....	59
2.2.4.3 PCR.....	60
2.2.4.4 ELECTROPHORETIC MOBILITY GEL SHIFT ASSAY.....	61
2.2.4.6 LABELLING OF THE CONSENSUS OLIGONUCLEOTIDE FOR NF- κB.....	62
2.2.4.7 SAMPLE PREPARATIONS.....	63
2.2.5 IMMONOPEROXIDASE (ABC) CYTOKERATIN STAINING OF CELLS FOR EPITHELIAL MARKERS.....	63
2.2.6 STATISTICAL ANALYSIS	64
Chapter 3 Epithelial characteristics of A549 and 16HBE cells and the role of GSH in cell proliferation.....	65
3.1 INTRODUCTION	65
3.2 CHARACTERISATION OF THE EPITHELIAL CELL LINES:	65
3.3 CELL CONFLUENCY.....	67
3.4 REDUCED GSH LEVELS IN THE EPITHELIAL CELL LINES.....	69
3.5 SUMMARY.....	74
3.6 DISCUSSION.....	74
Chapter 4 Induction of Nitric Oxide in Both A549 and 16HBE Cells and the Effect of Nitric Oxide Induction on GSH Levels.....	77
4.1 INTRODUCTION.....	77
4.2 INDUCTION OF NO [•] AND NITRITE PRODUCTION IN EPITHELIAL CELLS IN RESPONSE TO CYTOKINES AND LPS.....	77
4.3 INHIBITION OF NO [•] RELEASE BY THE NOS INHIBITOR LN ^G - MONOMETHYL-L-ARGININE (L-NMMA).....	84
4.4 INDUCTION OF NO [•] USING THE OXIDANT H ₂ O ₂	85
4.5 INTRACELLULAR GSH LEVELS FOLLOWING CYTOMIX AND H ₂ O ₂	89
4.6 PCR ANALYSIS OF iNOS.....	95
4.7 SUMMARY.....	98
4.8 DISCUSSION.....	99
Chapter 5 The Effect of Manipulating GSH Levels on NO[•] Induction and Release In Both A549 and 16HBE Cells.....	105

5.1 INTRODUCTION	105
5.2 MANIPULATION OF GSH LEVELS.....	105
5.3 THE EFFECT OF DEPLETING INTRACELLULAR GSH WITH BSO ON NO ⁻ PRODUCTION.	106
5.4 THE EFFECT OF INCREASING GSH LEVELS WITH THIOL COMPOUNDS ON NO ⁻ RELEASE.	112
5.5 THE EFFECT OF PRE-TREATING CELLS WITH THIOL COMPOUNDS ON CYTOMIX AND H ₂ O ₂ INDUCED NO ⁻ RELEASE.	117
5.6 PCR RESULTS.....	120
GAPDH.....	124
5.7 SUMMARY.....	125
5.8 DISCUSSION.....	125
Chapter 6 The effect of cytokines, H₂O₂ and modulating GSH levels on NF-κB activation.	130
6.1 INTRODUCTION	130
6.2 A TYPICAL GEL SHIFT.....	131
6.3 THE EFFECT OF CYTOMIX, INDIVIDUAL CYTOKINES AND LPS ON NF-κB ACTIVATION.....	134
6.4 THE EFFECT OF MODULATING INTRACELLULAR GSH LEVELS BY BSO AND THIOL COMPOUNDS ON NF-κB ACTIVATION.	138
6.5 THE EFFECT OF ROTENONE ON CYTOKINE AND H ₂ O ₂ INDUCED NF-κB ACTIVATION.....	146
6.6 THE EFFECT OF ROTENONE ON iNOS mRNA LEVELS.	154
6.7 INTRACELLULAR GSSG:GSH RATIO IN BOTH A549 AND 16HBE CELLS AT TIME POINTS WHEN NF-κB IS ACTIVATED.....	156
6.8 SUMMARY.....	158
6.9 DISCUSSION.....	159
7 Final Discussion.....	168
7.1 SUMMARY.....	168
7.2 CONCLUSIONS	169
7.3 TO CONCLUDE:	173
7.4 FUTURE STUDIES.....	174
Reference List.....	177

Chapter 1 Introduction

Oxygen was first characterised by Priestly and Lavoisier between 1772 and 1774 (Chinard, 1998) as a stable, odorless, tasteless and colourless gas. Oxygen is used in aerobic life form to oxidise carbon and hydrogen rich substrates, resulting in the chemical energy and heat essential for life. The major source of energy for most aerobic life arises from the reduction of oxygen to H_2O during respiration by the addition of 4 electrons. Reduction of oxygen during respiration can result in the formation of intermediates, which are both reactive and unstable and known as reactive oxygen species (ROS). ROS are generally harmful to their environment, but can be involved in physiological interactions such as the killing of micro-organisms (Bast et al., 1991) and the regulation of smooth muscle contraction (Bast et al., 1991).

The production of ROS is normally balanced in tissues by antioxidants, an imbalance generally resulting in oxidant induced injury and it is widely accepted that ROS play a role in the pathology of many diseases (Gutteridge, 1993).

Nitric oxide ($NO\cdot$) is a colourless gas and a weak reducing agent, first recognised as a gas by Priestly in 1772 (Gutteridge, 1994). It is a noxious chemical in the atmosphere but can be beneficial in the body in controlled doses. Recently there has been biological interest in $NO\cdot$ due to the observation that it is analogous to the endothelium derived relaxing factor (EDRF) (Ignarro et al., 1987; Palmer et al., 1987). Although a free radical, it is not as reactive as oxygen metabolites. At low levels it is beneficial, causing bronchodilation and smooth muscle relaxation (Barnes and Belvisi, 1993; Bast et al., 1991), but becomes cytotoxic at higher levels, forming peroxynitrite, a ROS (Ignarro and Murad, 1995) which decomposes to form a toxic hydroxyl-like radical (Gutteridge, 1994). $NO\cdot$ can therefore act as both an antioxidant and an oxidant.

There has been widespread research in the area of oxidants and the antioxidant defense system, as well as on the effects of NO[•] and its metabolites. The balance between ROS, antioxidants and NO[•] levels are important in disease states, especially in the bronchial and alveolar epithelium, and it is in these cells that the *in vitro* studies in this thesis were carried out.

The antioxidant GSH is a major focus of this study, since it plays a central role as a major protective intracellular and extracellular antioxidant. Its role in NO[•] induction/inhibition was investigated.

1. THE LUNG AND ROS.

The anatomy of the lung is important in the process of diseases due to both exogenous and endogenous oxidants. The following paragraph describes the anatomy of the lung.

1.1. STRUCTURE OF THE LUNGS.

The lungs are a system of symmetrical branching dichotomous tubes, starting at the mouth and nose and terminating at the alveolus, the ultimate unit of gaseous exchange. The airways can be divided into 2 portions, the upper airways consisting of the nose, nasal cavities, pharynx and larynx. The larynx is considered to be the boundary between the upper and lower respiratory tract (Jeffrey, 1998). The lower respiratory tract begins with the trachea and branching begins by division of the main bronchi, after which there are some 23 generations of branches before the alveoli are reached. There is progressive loss of cartilage, which is responsible for structural support, from the upper airways to the lower airways. The last bronchiolar divisions, which contain much less developed ciliated lining epithelium, are the terminal bronchi and lead into the respiratory bronchioles which have alveoli in the walls. The terminal bronchioles are the last of the solely conducting airways, the respiratory bronchioles and alveoli being involved in gaseous exchange. Support and elasticity

throughout the lung is provided by connective tissue, collagen and elastin which is found below the surface epithelium, and the presence of surfactant on the alveolar surface assists expansion of the lung during inspiration by reducing alveolar and bronchiolar surface tension. In the alveolar region the epithelium is in very close contact with the blood capillaries, favouring rapid and effective passage of gasses. The respiratory bronchioles, arising from one terminal bronchiole, together with its alveolar ducts and sacs, are known as the respiratory *acinus*, the basic unit of the lung (Jeffrey, 1998).

On average 10-15,000 litres of air pass through the lung per day (Jeffrey, 1998). The surface is therefore susceptible to inhaled irritants, and both infective and allergic substances. The airways therefore have protective mechanisms against such substances and also have mechanisms to condition the air before it reaches the respiratory portion of the lung. The conducting airways are involved in gas delivery to the alveoli, warming, humidifying and cleansing the air. A defence mechanism involving nervous reflexes (ie broncho-constriction or cough), ciliary action, mucous secretion and immune responses defends the lungs against pollution including irritants and bacteria etc (Jeffrey, 1996).

The airway and airspaces are lined by a single layer of cells, the epithelium, which are attached to a basement membrane. There are, however, many different epithelial cell types (Jeffrey, 1998), all having specific functions. These include ciliated cells for the removal of particles, serous cells and goblet cells for the production of mucus and clara cells which are important in metabolism and surfactant production. There is a progressive change in cell type from the upper airways to the lower airspaces. The alveoli contain two types of epithelial cells, the type I and type II pneumocytes. Most of the alveolar surface is covered by the more dominant type I epithelial cell which have many processes, each cell contributing to more than one alveolus. Their main function is to provide a barrier to prevent fluid loss and to facilitate gas exchange. Type I cells are connected to one another and to type II cells by tight junctions providing a selectively permeable barrier for fluid, molecules and ion movement between the interstitium and the alveolar space. Type II epithelial cells are cuboidal

in shape, and they only cover approximately 7% of the alveolar surface (Crapo et al., 1982). They are involved in the synthesis and secretion of the pulmonary surface active material, surfactant, and also act as progenitors of type I cells, regenerating a continuous epithelium after alveolar injury, such as that caused by oxidants. These two cell types are likely to differ in their antioxidant status, due to their function, and therefore have different susceptibility to oxidative damage (Persinger et al., 1996).

1.2 INFLAMMATORY CELLS

The experiments in this thesis use a mixture of cytokines, $\text{TNF-}\alpha$, $\text{IL1-}\beta$, and $\text{IFN-}\gamma$, collectively called cytomix. These pro-inflammatory cytokines are present during inflammation being released by both inflammatory cells and epithelial cells.

Cells that are important in the inflammatory response are present on the surface of the airspace epithelium cells at all times. These include mononuclear migratory cells such as intra-epithelial lymphocytes and mast cells, and alveolar macrophages (Crapo et al., 1983). A wider variety of inflammatory cells are, however, recruited during lung injury due to a variety of stimuli such as inhaled air pollutants (ozone, cigarette smoke, nitrogen dioxide and particles) and infection. Part of the cellular response to injury is the migration of leukocytes to sites of inflammation and injury, including macrophages, lymphocytes and polymorphonuclear (PMN) cells such as neutrophils. These inflammatory cells produce soluble mediators including proteins, interleukins and cytokines. Additionally neutrophils degranulate releasing lysosomal enzymes and other granule products. The function of phagocytic inflammatory cells is to kill bacteria and viruses and this is achieved, in part, by activation of the hexomonophosphate shunt to generate NADPH. The cells then utilize NADPH for the reduction of molecular oxygen, using the membrane bound flavoprotein cytochrome b-245 NADPH oxidase. This results in a 'respiratory burst', and the generation of superoxide anions, hydrogen peroxide, hydroxyl radicals and hypochlorous acid (Barnes, 1990; Kinnula et al., 1991; Root et al., 1975; Sedgwick et al., 1990), which are powerful anti-microbial agents.

Although inflammation is a defence mechanism against microbial infection, chronic inflammation has been associated with chronic lung disorders including asthma and adult respiratory distress syndrome (ARDS) (see later). Inflammatory cells are able to induce lung damage as well as being bactericidal, and a side effect of the respiratory burst, is an increase in oxidants which can damage lung cells as well as bacteria and viruses. In addition to an increase in oxidants, the low pH produced locally by phagocytes during inflammation may contribute to the release of transition metals, which enhance formation of the highly destructive hydroxyl radical (see later) (Halliwell et al., 1992). Hydrogen peroxide concentrations are also enhanced, being produced by lymphocytes, macrophages and PMNs. H_2O_2 is important not only as an oxidant but as a secondary messenger, activating immunologically important transcription factors such as NF- κ B, which switches on many genes for pro-inflammatory cytokines (Droge et al., 1994b; Winrow et al., 1993). Degranulation of neutrophils can also induce lung damage due to the release of elastases, which can cause lung damage if a protease/antiprotease imbalance occurs (Tetley, 1993). Inflammatory lung disorders are discussed in more detail later (see section 3 on inflammatory disorders).

1.3 REACTIVE OXYGEN SPECIES (ROS)

The effects of oxidants are examined in this study, in particular H_2O_2 , (which is present in the lower respiratory tract during inflammation), to determine their effects on NO \cdot induction and NF- κ B activation.

ROS are metabolites of molecular oxygen O_2 , and many of these oxidants are also free radicals. A free radical is defined chemically as *any species capable of independent existence that contains one or more unpaired electrons* (Halliwell, 1991a). Free radicals are usually unstable, reacting with a wide variety of compounds by either extracting or donating electrons, which results in stability of the radical, or by reacting with other free radicals, resulting in the formation of a covalent bond (Halliwell et al., 1992).

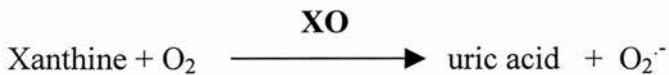
Respiration involves the reduction of bimolecular oxygen to water by a sequential 4-step addition of electrons.



A metabolic by product of respiration is the partial reduction of oxygen, by one electron, resulting in the formation of the superoxide anion ($\text{O}_2^{\cdot-}$):



The superoxide anion is also produced by the enzyme xanthine oxidase (XO), which converts water and xanthine in the presence of oxygen to uric acid and superoxide.



Recruitment and activation of inflammatory cells such as neutrophils and phagocytes also generate the superoxide anion during the respiratory burst.

The superoxide anion is rapidly converted to H_2O_2 by the enzyme, superoxide dismutase (SOD).



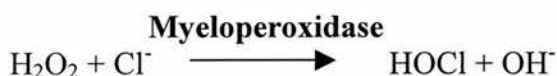
Hydrogen peroxide can also be formed by the action of the ferric iron on the superoxide anion (known as the Haber Weiss reaction).



Generation of H_2O_2 can result in the formation of the hydroxyl radical (OH^{\cdot}) non-enzymatically in the presence of Fe^{2+} in a secondary reaction, known as the Fenton reaction.



Neutrophils also produce the enzyme, myeloperoxidase, which converts hydrogen peroxide and chloride to hypochlorous acid (HOCl).



These oxidants, H_2O_2 , OH^{\cdot} , $\text{O}_2^{\cdot-}$ and HOCl are typical of those produced during inflammation by migratory leukocytes such as macrophages and neutrophils and are important in the process of bacterial killing by phagocytes.

Another oxidant which may be present in the lung during inflammation, is NO^{\cdot} and its metabolites. NO^{\cdot} , a free radical, can be induced by cytokines and released by both epithelial cells (Adcock et al., 1994; Felley-Bosco et al., 1994; Robbins et al., 1994) and inflammatory cells such as neutrophils and macrophages, (Kinnula et al., 1995; Marletta et al., 1997). It can react readily with the superoxide anion resulting in the formation of peroxynitrite (ONOO^{\cdot}) a highly toxic free radical (Kinnula et al., 1995).



Peroxynitrite can then decompose into other oxidants, to release hydroxyl like radicals, independent of metal catalysis.



Lung epithelial cells also generate oxidants but the action of these oxidants is normally limited within the cell by antioxidants, which effectively remove oxidants before they cause intracellular damage (Kinnula et al., 1992; Kinnula et al., 1991). They are therefore not generally released, unlike inflammatory cells, which release oxidants in order to kill bacteria. However, some epithelial cells do release oxidants into the extracellular space of the lung, such as alveolar type II cells, which release

H₂O₂ (Barnes, 1990). H₂O₂ is fairly stable and not particularly damaging to normal tissue and acts as a secondary messenger in the activation of the transcription factor NF- κ B (see later). The injurious potential of hydrogen peroxide is, however, amplified by conversion to the hydroxyl radical which is a potent oxidant, capable of causing damage to other cells. The presence of the superoxide anion further potentiates this reaction, by converting ferric iron (Fe³⁺) to ferrous iron (Fe²⁺) in the Haber Weiss reaction, thus enabling the Fenton reaction to occur.

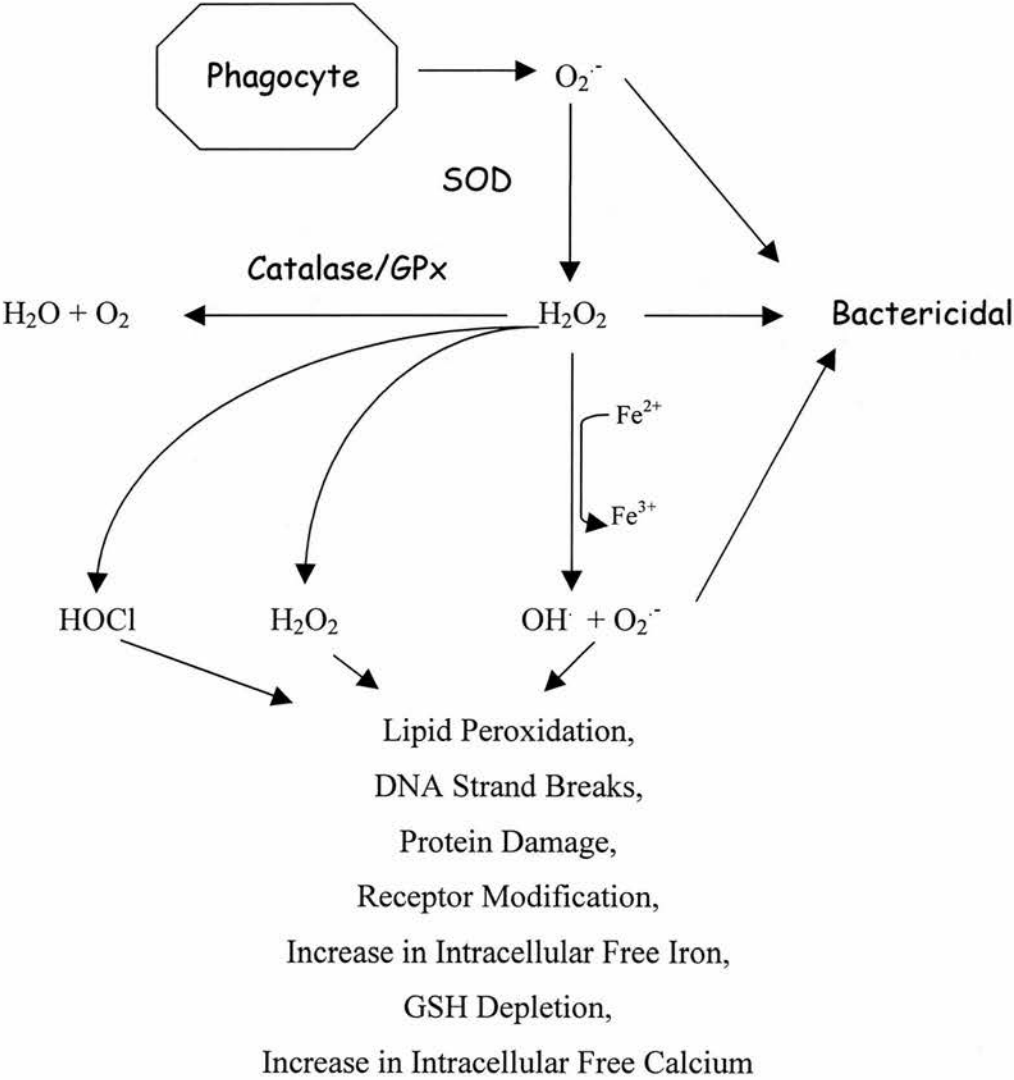
1.4 ROS AND OXIDATIVE DAMAGE.

Oxidative stress results from an imbalance in the oxidant/antioxidant equilibrium in favour of oxidants and relevant reactive oxygen species (ROS). In the lung ROS include, O₂⁻, OH[•], H₂O₂, HOCl and ONOO⁻, all of which are important players in cell and tissue injury during inflammation, being produced by several types of inflammatory cells (Barnes, 1990; Halliwell et al., 1992). ROS have been implicated in several lung diseases such as asthma (Barnes, 1990), chronic obstructive pulmonary disease (COPD) (Kinnula et al., 1995), smoking related emphysema (Heffner and Repine, 1989) and the alveolar disease adult respiratory disorder (ARDS) (Brigham, 1990), where they cause both tissue and cell damage.

ROS can exert different toxic effects during oxidative stress causing injury to the airways in a number of ways (Barnes, 1990). These include modification of receptor activity and signaling (Cochrane, 1991) by oxidation of amino acids such as methionine and cysteine; release of endogenous mediators of inflammation by acting as signaling mediators, (Baeuerle et al., 1996; Pahl and Baeuerle, 1994) resulting in the over expression of normal physiological processes; and acute cell death and tissue injury, which is often mediated by the hydroxyl radical (Farber et al., 1990). It has also been suggested that oxidants and the redox status of the cell are important in the mechanisms of cell death by both apoptosis and necrosis (Kazzaz et al., 1996). Both cellular and plasma proteins can be damaged undergoing cross-linking and aggregation (Wolff et al., 1986) in the presence of free radicals. The cell membrane undergoes lipid peroxidation due to a high content of polyunsaturated fatty acids

which provides an electron rich environment which is highly susceptible to oxidative attack. Lipid peroxidation causes a decrease in the fluidity of the membrane, destabilising membrane receptors and products of lipid peroxidation, primarily aldehyde derivatives, which can inhibit protein synthesis, block macrophage action and cause changes in chemotaxis and enzymatic activity (Bast et al., 1991). Lipid peroxidation also occurs in the mitochondrial membrane resulting in loss of energy and cellular stores of ATP, and damage of mitochondrial DNA (Schraufstatter et al., 1986). Both single and double stranded DNA is damaged, accompanied by hydroxylation of bases following oxidant or radical attack (Halliwell and Arouma, 1991b) (Ryrfeldt et al., 1993). Oxidant stress within the cell also causes a change in the redox state of the cell, in particular glutathione, which is oxidised (to GSSG), and can cause damage resulting in the release of calcium from its intracellular stores (Wefers and Sies, 1989). A vicious circle of injury occurs on release of iron and other transition metals due to oxidative stress, which enhances hydroxyl radical formation. Figure 1 shows a schematic diagram of the formation of ROS, and the damage they can cause.

Figure 1: The Formation of ROS



2 Antioxidants

This section describes the antioxidant systems which allow the protection of cells against oxidant induced injury. GSH is discussed in more detail separately, since this was the antioxidant studied in this thesis.

An antioxidant is defined as *any substance that, when present at low concentrations compared to those of the oxidisable substrate, significantly delays or inhibits the oxidation of that substrate* (Gutteridge, 1994). There are different categories of

antioxidant effects (Heffner and Repine, 1989), which often have overlapping properties:-

1. The prevention and compartmentalisation of free radical formation.
2. The conversion of oxidants to less reactive species
3. The repair of oxidant-induced injury.

2.1 THE PREVENTION AND COMPARTMENTALISATION OF FREE RADICALS.

The prevention and compartmentalisation of free radicals is primarily achieved by the 4 electron reduction of oxygen to water by cytochrome oxidase in the mitochondria. The mitochondria utilise more than 90% of the oxygen in lung cells and metabolise it in a way to avoid significant free radical formation (Heffner and Repine, 1989).

2.2 THE CONVERSION OF OXIDANTS TO LESS REACTIVE SPECIES.

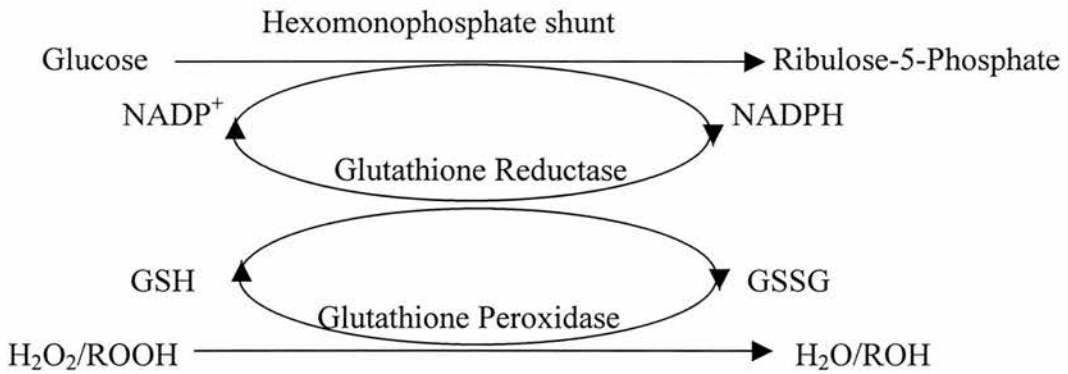
Oxidants are converted to less reactive species by 3 major intracellular and extracellular enzymes which provide a critical defence mechanism (Heffner and Repine, 1989; Kinnula et al., 1995) including:

- Catalase, which catalyses the degradation of hydrogen peroxide to water and oxygen, mainly within peroxisomes.
- Superoxide dismutases which catalyse the dismutation of superoxide anion to hydrogen peroxide. The superoxide dismutases are a family of enzymes with different intra and extracellular distributions. They include copper zinc SOD (CuZnSOD), which is found mainly in the cytosol, but also is found in the nucleus and manganese SOD (MnSOD), which exists in the mitochondria, eliminating superoxide anions produced during electron transport. The extracellular copper SOD (ECSOD), is found in the epithelial lining fluid of the lower respiratory tract. The SODs are thought to play a protective role in cells that produce both superoxide anion and nitric oxide since by dismutating the

superoxide anion they also prevent the formation of the toxic peroxynitrite, by preventing superoxide anions from reacting with NO \cdot , and thus prolong the bioavailability of NO \cdot as an antioxidant (see 2.5) (Beckman et al., 1990).

- Glutathione peroxidase (GPx), which reduces hydroperoxides to water, using GSH as a reducing substrate, which is, itself, oxidised to a GSH dimer, GSSG. GPx is part of the GSH redox system (figure 2) having a central role in the reduction of intracellular hydroperoxides, complementing catalase in lowering H₂O₂ levels. GPx is also able to eliminate hydroperoxides such as lipid peroxide, formed during lipid peroxidation. GSH is the reducing substrate for the enzyme and GSSG is the product, GSSG is then reduced by glutathione reductase (GR) using NADPH as a reducing agent. NADPH is supplied via the activity of glucose-6-phosphate in the hexomonophosphate shunt.

Figure 2. The Glutathione Redox Cycle.



As well as these enzyme systems, there are a whole array of molecules which act as sacrificial antioxidants by scavenging free radicals directly. Examples are given in table 1 (Heffner and Repine, 1989)

Table 1: Oxidant And Free Radical Scavengers (Heffner and Repine, 1989).

Scavenger	Structure	Tissue site	Actions
Vitamin E	Fat soluble vitamin	Lipid membranes, extracellular fluids	Converts $O_2^{\cdot-}$, OH^{\cdot} and lipid peroxy radicals to less reactive forms, breaks lipid chain reactions
B-Carotene	Metabolic precursor to vitamin A	Tissue membranes	Scavenges $O_2^{\cdot-}$, reacts with peroxy radicals
Bilirubin	Product of hemoprotein catabolism	Bloodstream, tissue	Chain breaking antioxidant, reacts with ROO^{\cdot}
Vitamin C	Water soluble vitamin	Wide distribution – intracellular and extracellular	Scavenges $O_2^{\cdot-}$ and OH^{\cdot} neutralises oxidants from stimulated neutrophils
Uric Acid	Oxidised purine base	Wide distribution	Scavenges OH^{\cdot} , $O_2^{\cdot-}$ and peroxy radicals, prevents oxidation of vitamin C
Glucose	Carbohydrate	Wide distribution	Scavenges OH^{\cdot} .
Cysteine	Amino acid	Wide distribution	Reduces organic compounds by donating electron from sulphhydryl group
Cysteamine	Amino acid	Wide distribution	Same as cysteine
Taurine	B- α amino acid	Radical generation	Conjugates xenobiotics, reacts with $HOCl$
Albumin	Protein	Upper airways	Reacts with oxidants as a sacrificial antioxidant
Tracheobronchal mucus	Protein and glycoprotein	Wide distribution	Scavenges inhaled oxidants

2.3 REPAIR OF OXIDANT INDUCED INJURY

Repair of oxidant induced injury by antioxidants is not well understood but involves removal of injured cellular components, activation of nuclear enzymes for DNA repair and induction of cellular proliferation (Heffner and Repine, 1989).

2.4 EXTRACELLULAR ANTIOXIDANT DEFENCES.

Both conducting and respiratory airways have a liquid covered surface, and the integrity of the fluid balance of this surface layer is necessary for maintaining a protective environment as well as maintaining optimal gas exchange. In the respiratory epithelium, this layer of fluid is referred to as the epithelial lining fluid (ELF), which is rich in antioxidants. These include Vitamin E and C, ceruloplasmin, transferrin, ascorbate, albumin, methionine and GSH (Cantin et al., 1987; Davis and Pacht, 1998; Matalon et al., 1990). The lung is unique in that the extracellular milieu at the alveolar epithelial surface, the ELF, is rich in GSH (Cantin et al., 1987). GSH and other antioxidants are thought to either, be secreted by cells (Cantin et al., 1987) or to diffuse from the blood (Heffner and Repine, 1989), platelets and erythrocytes being rich in antioxidants and having the potential to be recruited during inflammation. Certain lung cells are capable of exporting GSH (Cantin and Begin, 1991; Deneke and Fanburg, 1989) and the alveolar macrophage is also a candidate, containing 9 times more GSH than other lung cells (Cantin and Begin, 1991; Horton et al., 1987). Macrophages contain high levels of GSH in order to protect themselves against the hydrogen peroxide they release during phagocytosis (Root et al., 1975). A striking feature of the ELF are the surprisingly low levels of oxidised GSH, GSSG, despite the potentially large oxidant burden, thus implying an effective mechanism, possibly involving γ -GT, for removal of GSSG by degradation, reduction or both (Cantin and Begin, 1991). Both macrophages and type II alveolar cells exhibit γ -GT activity (Liu et al., 1996), which is possibly involved in the degradation of GSSG and the uptake of its corresponding amino acids for de novo synthesis of GSH within the cell (see section 5).

Table 2 shows the concentrations of antioxidants in the ELF and compares them to both the plasma and the nasal lining fluid.

Table 2: A comparison of antioxidants present in the plasma, the ELF and the nasal lining fluid (Cross et al., 1994)

Antioxidant	Plasma μM	ELF μM	Nasal lining fluid μM
Ascorbic acid	40	100	40
Glutathione	1.5	100	40
Uric acid	300	90	160
α -Tocopherol	25	2.5	-
Albumin SH	500	70	10

2.5 NO \cdot AS AN ANTIOXIDANT.

NO \cdot , is a small, relatively unstable, free radical, that readily crosses lipid membranes where it interacts with specific targets for cell signaling. It can exert both beneficial and deleterious effects in different tissues under different conditions, being able to act as both an oxidant and an antioxidant. NO \cdot can be produced by 2 different enzymes, one is constitutive and the other inducible (Ignarro and Murad, 1995). Constitutive NO \cdot is produced in regulated amounts and is likely to act as an antioxidant. The endothelium derived relaxing factor now recognised as NO \cdot , relaxes smooth muscle by activation of soluble guanylyl cyclase in adjacent cells and has been shown to inhibit leukocyte adhesion, a rate limiting step in inflammation, possibly by scavenging superoxide anion (Barnes, 1993; Granger and Kubes, 1996). NO \cdot can react rapidly with hydroperoxyl radicals, and therefore prevent lipid peroxidation (Rubbo et al., 1994). It is highly lipid soluble and can partition into the membrane where it can act as a chain terminator of radical-mediated lipid peroxidation. Virtually any radical species formed in or near the lipid membrane can react with NO \cdot (Crow and Beckman, 1995). NO \cdot has also been shown to inhibit

superoxide anion production by neutrophils (Clancy et al., 1992). This is not due to its scavenging properties, but due to direct effects on the membrane component of NADPH oxidase thus preventing the respiratory burst. This is likely to occur following cytokine induced NO[•] release by cells such as macrophages at sites of inflammation, so protecting against tissue injury. NO[•] can therefore act as an antioxidant, but becomes pro-oxidant after reacting with the superoxide anion, resulting in peroxynitrite, which is produced in great amounts during inflammation (Rubbo et al., 1994).

3 Inflammatory Disorders Involving ROS And Antioxidants.

Oxidants are thus increased during inflammation and a variety of antioxidants are present in both lung cells, and the ELF, that are important in the safe removal of oxidants. However, in cases of extreme oxidative stress, such as that produced during the respiratory burst, an oxidant/antioxidant imbalance may occur, resulting in both cell and tissue injury. Several lung disorders are associated with increased inflammation, and it is thought that oxidants play a role in lung damage in these disorders. It is therefore possible that an oxidant/antioxidant imbalance is occurring in several of these disorders which are described below.

3.1 ASTHMA

Asthma is a chronic inflammatory disease of the conducting airways, and can be characterised by:

- Variable and reversible airflow obstruction,
- Bronchial hyperresponsiveness, defined as an excessive airway narrowing in response to various stimuli.

The earliest reported histological features of asthma (Dunhill, 1960) are epithelial damage, thickening of the reticular basement membrane and infiltration of the airway wall by mononuclear cells and eosinophils. More recently broncho-alveolar lavage

studies indicate that asthmatic airways undergo an inflammatory response involving activated T-helper cells, lymphocytes, eosinophils and mast cells (Azzawi et al., 1990; Azzawi et al., 1992; Holgate, 1993). The disordered function is due to the secretion of an array of vaso-bronchoactive mediators (Busse et al., 1993), which interact in a complex way, resulting in the pathophysiology of asthma. Epithelial cells are also a source of pro-inflammatory cytokines and other mediators such as NO[•] (Barnes, 1996), which contribute to the disease. Thus it has become clear that an inflammatory response is a key element in the development of asthma. Airway narrowing and airflow obstruction, which are reversible either spontaneously or by treatment, result in the symptoms of dyspnoea and wheezing and increase airway responsiveness to a variety of stimuli (Holgate, 1993). Airway hyperreactivity or hyperresponsiveness, although not unique to asthma, is therefore a hallmark of asthma.

An increased oxidant burden, in particular from the release of superoxide anions from activated macrophages (Calhoun et al., 1992) and hypodense eosinophils (Sedgwick et al., 1990) has been detected in asthmatic patients following an allergen challenge. Both alveolar macrophages and BAL from asthmatic patients release more free radicals compared to control patients' (Kinnula et al., 1995), and this increased oxidant burden may contribute to airway injury. Free radical generation correlates negatively with lung function (Kinnula et al., 1995) and epithelial loss correlates with airway reactivity in asthmatics (Jeffrey et al., 1989). Moderate to severe asthmatic patients have decreased levels of antioxidants (Novak et al., 1991), such as catalase and GSH in plasma and red blood cells, but mild asthmatics have increased antioxidant defences in their BAL, possibly to counterbalance an increase in oxidant generation and prevent airway injury (Smith et al., 1993).

NO[•] has also been detected in the breath of asthmatics in higher levels than those found in normal subjects (Byrnes et al., 1997; Massaro et al., 1995) and has been suggested as a clinical marker for airway inflammation. Although much of the exhaled NO[•] is thought to be a product of the nasal passage, it has been shown that increased NO[•] in the breath of asthmatics arises from the lower airways (Kharitonov

et al., 1996). Inhalation of an inhibitor of the inducible nitric oxide synthase (iNOS) in asthmatics decreases exhaled NO, suggesting that increased NO in the breath is due to induction of iNOS (Yates et al., 1996). mRNA for NOS has been found in airway epithelial cells, macrophages, neutrophils, eosinophils and mast cells (del Pozo et al., 1997; Gaston et al., 1994), and iNOS is synthesised on stimulation with inflammatory cytokines (Barnes and Belvisi, 1993) (see later). Although NO can be beneficial to the airways in small amounts, causing relaxation of vascular and airway smooth muscle, it can become cytotoxic at higher levels. NO can react with the superoxide anion resulting in the formation of the oxidant peroxynitrite (ONOO-), which although it contributes as a defence mechanism, being cytotoxic to bacteria, it also contributes to the oxidant burden, causing cell damage. Thus it is established that an increased oxidant burden is present in asthmatics and that bronchial epithelial damage is a component of the injurious inflammatory response (Jeffrey et al., 1989). The antioxidant status of the bronchial epithelium may therefore be an influencing factor in the susceptibility of individuals to the effects of an inflammatory response following exposure to allergens and thus the subsequent development of an asthma attack. Thus antioxidants should have a role in the prevention or treatment of the condition.

3.2 ARDS

ARDS, which was first recognised 30 years ago (Ashbaugh et al., 1967), is a disorder which affects the gaseous exchanging region of the lung, the alveolar capillary membrane, resulting in acute lung injury and respiratory failure. It is a condition that is associated with mortality as high as 60%. It is characterised pathologically (Cochrane et al., 1983) by an influx of neutrophils into the lungs, alveolar damage, hypoxaemia, and bilateral pulmonary infiltrates on chest x-ray associated with increased alveolar and capillary permeability, non-cardiogenic pulmonary oedema and decreased lung compliance. It may occur due to insults to the lung, such as pneumonia, but may also result from an extra pulmonary cause of shock, such as

trauma and sepsis. Death is often due to non-pulmonary causes as a result of sepsis and multi organ failure (Dantzker, 1990).

There is evidence that ARDS is associated with oxidative stress, leading to an oxidant/antioxidant imbalance (Brigham, 1990; Cochrane et al., 1983). Increased oxidants are also thought to cause inactivation of antiproteases in the alveolar space (Cochrane et al., 1983), producing the potential for proteolytic injury. Decreased levels of antioxidants such as GSH and higher levels of GSSG are found in the alveolar ELF of patients with ARDS (Bunnell and Pacht, 1993; Pacht et al., 1991), suggesting that oxidant stress has occurred in the lower respiratory tract. A deficiency in GSH is thus thought to be important in the pathogenesis of the disease.

Increased NO^\cdot levels have been found in the broncho-alveolar lavage, in animal models of ARDS, following exposure to endotoxin (Pheng et al., 1995). iNOS mRNA has also been found in human lung biopsies and in the lungs at autopsies of ARDS patients (Sachdev et al., 1997). The level of nitrotyrosine residues (produced by the action of peroxynitrite) have been monitored in ARDS patients and these residues were found to be increased with increased disease severity (Kooy et al., 1995; Royall and Kooy, 1997). The presence of NO^\cdot and peroxynitrite in ARDS in humans is controversial, but is likely to be present during severe inflammation. NO^\cdot is, however, often used, in low doses, in the treatment of ARDS, to improve oxygenation (Johannigman et al., 1997; Yoshida et al., 1997), but this treatment, along with oxygen treatment, may only be supportive, and may potentiate further injury.

3.3 OTHER RESPIRATORY DISORDERS ASSOCIATED WITH INCREASED ROS.

There are several other respiratory disorders which have been reported to be due, at least in part, to an increased oxidant burden caused by the inflammatory response and an imbalance in the oxidant/antioxidant status of the lung. These include:

- *Idiopathic Pulmonary Fibrosis (IPF)* is characterised by an accumulation of alveolar macrophages and neutrophils in the lower respiratory tract, cell injury, fibrosis of the alveolar wall and a loss of lung architecture (Crystal, 1991; Saleh et al., 1997). A 4-5 fold increase in inflammatory cells is observed in bronchoalveolar lavage (macrophages, neutrophils), as are increases in cytochrome b-245, resulting in an increased oxidative burst which mediates the process of cell injury (Saleh et al., 1997). There is a 3-fold decrease in GSH in the ELF, thus adding to the antioxidant/oxidant imbalance. Both iNOS and eNOS have been observed by immunohistochemistry in lung biopsies of IPF patients at levels much higher than those observed in normal lungs (Saleh et al., 1997). IPF is also associated with increases in nitrotyrosine, as observed by immunostaining of lung biopsies from both IPF and normal lungs (Saleh et al., 1997). Increased nitrotyrosine residues were observed in airway epithelium, alveolar epithelium, macrophages and neutrophils. This suggests that increased production of NO[•] and peroxynitrite may be partly responsible for the damage observed in this disease.
- *Cystic fibrosis (CF)* is a genetic disease, which mainly affects the lungs and gastrointestinal tract. In CF the lung contains thick, infected mucus, and there is chronic inflammation dominated by neutrophils and macrophages. Injury is thought to occur due to the increased oxidant burden caused by chronic infections (Crystal, 1991), eventually resulting in respiratory failure. Increased NO[•] levels are not observed in the breath of cystic fibrosis, but this is possibly due to the thickness of mucus preventing NO[•] diffusion (Balfour-Lynn et al., 1996), which is further supported by the fact that nasal NO[•] in CF patients is decreased compared to normal subjects (Lundberg et al., 1996).
- *Chronic obstructive pulmonary disease (COPD)* results largely from the inhalation of cigarette smoke, which contains 10¹⁷ oxidants/puff (Pryor et al., 1976). Chronic bronchitis and emphysema are the major conditions that make up COPD (Jeffrey, 1992). Both disorders are associated with increased alveolar macrophage and neutrophil recruitment in the BAL. Emphysema is associated with an increase in the size of the alveolar spaces distal to the terminal bronchioles (Flenley et al., 1986) due to the degradation of elastin by proteases such as elastase, or oxidants which are found in cigarette smoke or released by

the influx of inflammatory cells (Riley and Kerr, 1998). Cigarette smoke also leads to further recruitment of PMNs, which release proteolytic enzymes such as elastases (Flenley et al., 1986). There is therefore an increase in oxidant burden from both inhaled oxidants and oxidant release from inflammatory cells. These oxidants are thought to inactivate anti-proteases such as α_1 -antitrypsin, causing a protease/antiprotease imbalance leading to proteolytic damage to matrix structures such as elastin in the alveolar walls resulting in the airspace enlargement that is emphysema (Tetley, 1993). An oxidant/antioxidant imbalance is also thought to occur in this condition (Rahman and MacNee, 1996) and it is likely that NO^\cdot and peroxynitrite also play a role.

Evidence thus suggests that inflammatory lung diseases are associated with increased oxidative stress (Cochrane et al., 1983; Crystal, 1991) resulting from an oxidant/antioxidant imbalance. Administration of compounds to augment the antioxidants found in the ELF may therefore be beneficial in preventing oxidant-induced damage.

4 Nitric Oxide (NO^\cdot)

The main focus of this thesis is the induction of nitric oxide by a variety of stimuli including cytokines and oxidants, and the effect of the redox state of the cell, particularly glutathione, on this induction. This section provides background information on NO^\cdot , its induction and molecular regulation and the effect of NO^\cdot on its environment.

4.1 BACKGROUND

In 1992, the journal *Science* declared NO^\cdot to be molecule of the year (Culotta and Koshland, 1992). NO^\cdot is unusual in that it is a noxious chemical in the atmosphere but in controlled concentrations in the body it can be beneficial. It is a relatively

unstable, uncharged free radical that reacts readily with acceptor molecules. A role for NO^\cdot in biological reactions was discovered in 1987 by Ignarro and colleagues (Ignarro et al., 1987) and Palmer and colleagues (Palmer et al., 1987) when it was shown to have the same biological properties as endothelium derived relaxing factor (EDRF), the endogenous stimulator of guanylate cyclase. EDRF was discovered in 1980 by Furchgott and Zawadzki (Furchgott and Zawadzki, 1980) when it was demonstrated that vascular relaxation induced by acetylcholine (ACh) depended on the presence of the endothelium, providing evidence that this effect was mediated by a labile humoral factor, which was called EDRF. EDRF was shown to be activated by a variety of stimuli such as acetylcholine, thrombin, bradykinin and hypoxia (Furchgott and Zawadzki, 1980). However agents such as nitrovasodilators and β -adrenergic agonists also stimulated EDRF independent of the presence of the endothelium (Ignarro et al., 1987). EDRF was shown to be NO^\cdot using a chemiluminescence assay and its release was proportional to the amount of bradykinin required for EDRF activation (Ignarro et al., 1987). Further comparisons of the effects of EDRF and NO^\cdot using vascular strips provided evidence that the two were indistinguishable (Marletta, 1989; Moncada et al., 1991). Superoxide anions appear to contribute to the instability of EDRF since the effects of NO^\cdot could be prolonged by the addition of superoxide dismutase (SOD) (Gryglewski et al., 1986) which dismutates superoxide anions to hydrogen peroxide, thus preventing the creation of NO^\cdot with the superoxide anion. Simultaneously with the discovery that EDRF was analogous to NO^\cdot , two groups investigating the mechanisms of murine macrophage-induced cytotoxicity attributed this activity to NO^\cdot (Hibbs et al., 1987; Iyengar et al., 1987).

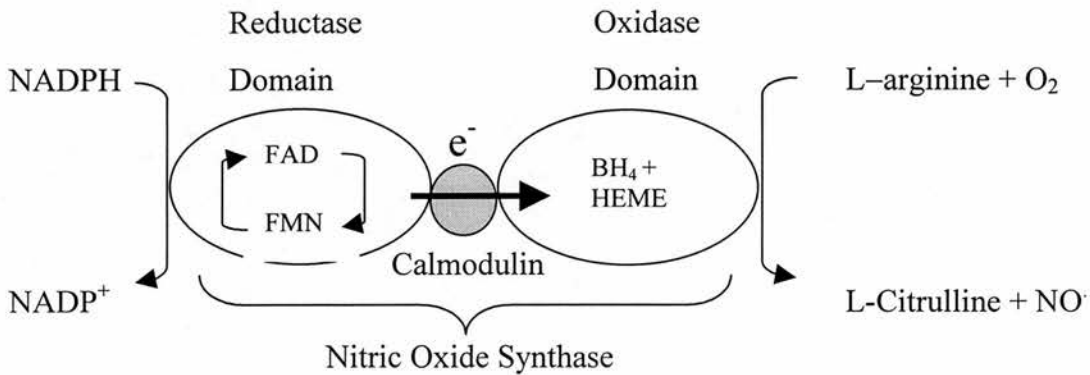
4.2. METABOLISM/BIOCHEMISTRY

NO^\cdot is formed by the conversion of L-arginine to L-citrulline, requiring bi-molecular oxygen and NADPH. The requirement of L-arginine for NO^\cdot formation was discovered in macrophages by Hibbs and colleagues (Hibbs et al., 1987), when it was shown that the cytotoxic effects of macrophages on DNA synthesis and mitochondrial respiration was due to NO^\cdot and was dependant on L-arginine. This

was further supported by Palmer and colleagues in 1988 (Palmer et al., 1988) who showed that NO[•] release from endothelial cells required L-arginine. The enzyme responsible for NO[•] production is known as nitric oxide synthase (NOS) and there are three types; Neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial constitutive NOS (ecNOS) (Ignarro and Murad, 1995) all of which are highly homologous to each other. There are, however differences in expression and activation. Both nNOS and eNOS are constitutively expressed proteins that require enzyme activation by a calcium/calmodulin signaling pathway, an increase in calcium levels resulting in the binding of calcium to calmodulin on the enzyme followed by activation of the enzyme. iNOS, however, requires de novo mRNA and protein synthesis. The enzyme causes the release of massive amounts of NO[•], independent of calcium levels. It is the inducible enzyme that is of interest in this thesis since we are inducing NO[•] in cells by cytokines and oxidants.

Inducible NOS is a homodimeric protein which shares homology in its C terminal with the heme protein NADPH cytochrome P-450 reductase (CPR) (White and Marletta, 1992). NOS catalyses the production of NO[•] by a five electron oxidation of the terminal guanidino nitrogen of L-arginine (Stamler et al., 1992). Arginine oxidation by NOS consists of 2 monooxygenation reactions resulting in an intermediate NO-hydroxyl-L-arginine. The enzyme consumes 2 moles of O₂ and 1.5 moles of NADPH per mole of NO[•] formed. Therefore for catalysis, NOS must bind and activate oxygen twice, in tight proximity to the L-arginine binding active site, using electrons derived from NADPH. Figure 3 shows the steps involved in NO[•] production (Marletta, 1993)

Figure 3: Schematic view of NOS, the interaction of the flavin reductase and oxidative heme domain (Marletta, 1993).



The formation of NO[•] is both achieved and regulated by 5 bound cofactors and prosthetic groups present on the enzyme. These include FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), a heme group (iron protophorphyrin IX), BH₄ (tetrahydrobiopterin) and calmodulin. NOS contains both a reductase and an oxidase domain. The reductase domain serves two purposes; as a conduit for delivering single electrons to molecular O₂ from NADPH ie electrons flow from NADPH to FAD to FMN, and to act as an electron reservoir. Between the two domains there is an interdomain which contains a calmodulin binding sequence. In iNOS this sequence has an extremely high avidity for calmodulin, ensuring that calmodulin is virtually always bound to the enzyme, independent of calcium levels. Calmodulin binding to iNOS is able to regulate enzyme activity by triggering an electron flux from FMN to the heme group ie it couples the oxidase and reductase domains. Both arginine and BH₄ bind to the oxygenase domain, BH₄ is thought to be an active cofactor, activating O₂ for catalysis (Marletta, 1993).

iNOS is induced in almost every tissue and cell type. Its predominate mechanism of induction is by transcriptional regulation. Low basal rates of inducible NO[•] formation are enhanced by cytokines and/or LPS but maximal induction occurs with a synergistic combination of the proinflammatory cytokines such as TNF- α , IL1- β ,

IFN- γ and LPS in many cell types including epithelial cells, endothelial cells, hepatocytes and kupffer cells (Ignarro and Murad, 1995).

iNOS is found on chromosome 17, consists of 26 exons, 25 introns and is a 37KB genomic DNA (Chartrain et al., 1994). Sequence analysis of the 5' flanking region revealed consensus sequences important in cytokine modulation of gene expression, including NF- κ B, IRF-1, the IFN- γ responsive element, NF-interleukin 6 (IL6), and TNF-RE like sites (Chartrain et al., 1994; De Vera et al., 1996). The promoter region for the gene has been shown to interact with NF- κ B (Xie et al., 1994) (Ignarro and Murad, 1995) which is thought to be critical in the induction of iNOS. More distal regions of the promoter may however be involved in IFN- γ stimulation of iNOS induction. These include the IRF-1 cis region of the DNA since IRF-1 knockout mice do not exhibit an iNOS response (Ignarro and Murad, 1995) suggesting that although NF- κ B activation is necessary for NO \cdot induction, interaction with other transcription factors may be required (Salkowski et al., 1996).

4.3 NO \cdot AS A FREE RADICAL.

NO \cdot is a small hydrophobic molecule, being more soluble in hydrophobic solvents than in aqueous solutions, and is therefore suitable to be both an intra- and extracellular messenger. NO \cdot is able to move by random motion, having a half-life of between 5-15 seconds and can reach the cell membrane within 2-30 milli seconds. NO \cdot can react in the cell in two ways; by autoxidation (+ O₂) and by reacting with major cell species such as metal ions and superoxide. As NO \cdot is synthesised, it spreads out until the rate of appearance (synthesis and diffusion) and disappearance (e.g. autoxidation) counterbalance each other, a steady state being reached in about 20 seconds.

NO \cdot is uncharged but has an unpaired electron and as such is a free radical. It therefore reacts with other species having unpaired electrons, resulting in secondary products which are often more reactive. NO \cdot reacts rapidly with bi-molecular oxygen in a process of autoxidation:



Nitrogen dioxide (NO_2) further reacts with NO^\cdot to give dinitrogen trioxide (N_2O_3).



N_2O_3 further reacts to give nitrite (NO_2^-):



NO also reacts with transition metals and other free radicals such as the superoxide anion:



Peroxynitrite is relatively stable but will be protonated at physiological pH to yield peroxynitrous acid (ONOOH), which is unstable and will rapidly decompose to yield NO_3^- via the formation of an intermediate with hydroxyl like properties:



Thus NO^\cdot release can result in the formation of the fairly stable and unreactive nitrate (NO_3^-) and Nitrite (NO_2^-), the very reactive peroxynitrite (ONOO^-) and nitrogen dioxide (NO_2). These products of NO^\cdot induction and release result in a variety of effects, including nitrosation of amines, thiol containing peptides and proteins, lipid peroxidation, and the formation of complexes with transition metals, such as metalloproteins and haemoglobins (Ignarro and Murad, 1995; Robbins and Grisham, 1997).

Nitrite and nitrate are relatively unreactive end-products of NO^\cdot oxidation, but intermediates such as nitrogen dioxides and peroxynitrite, can be involved in lipid

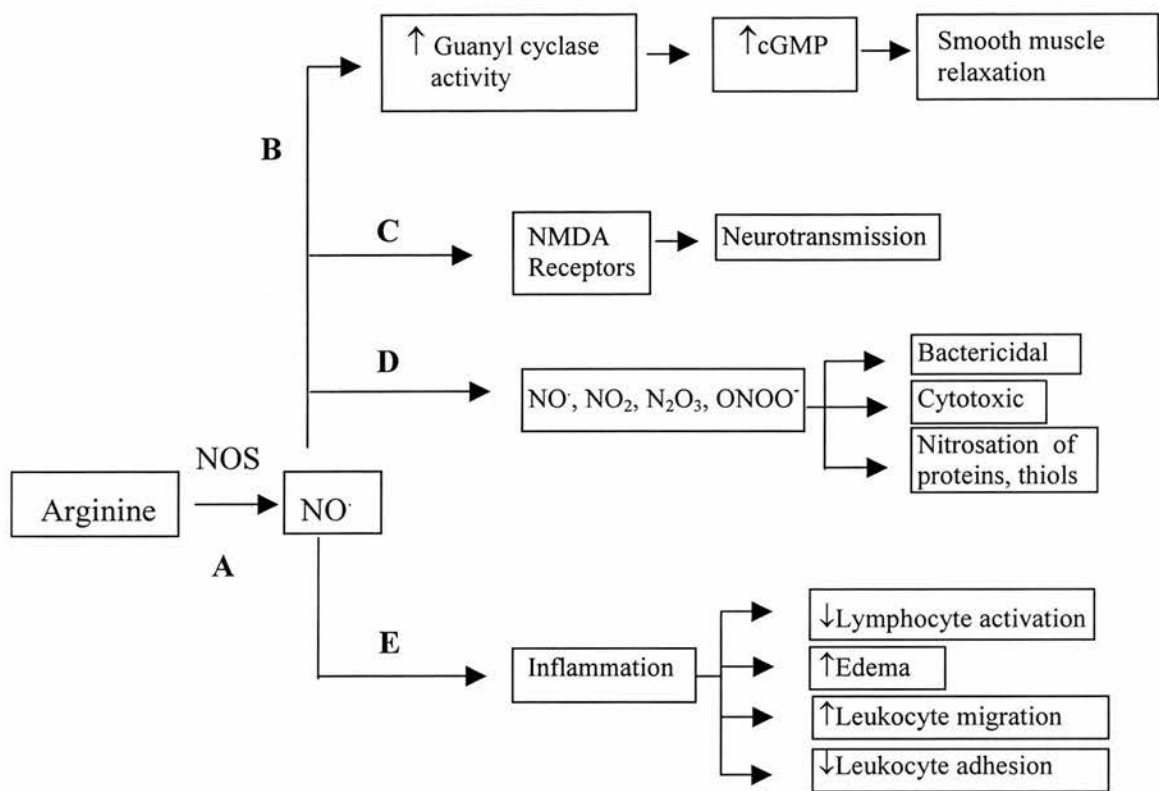
peroxidation and nitrosation, which would account for the high concentrations of RS-NO products found in the lungs during inflammation (Gaston et al., 1993) (Stamler, 1997). Nitrosation involves the reaction or transfer of NO^+ and forms a relatively stable product. NO^\cdot is able to react with GSH in aerobic conditions forming S-nitrosothiols, (GSNO) (Hogg et al., 1996). One of the main products of nitrosation are nitrosothiols, the S and N providing stability. Formation of nitrosothiols provide a means of stabilising NO^\cdot in a bioactive form for transport within tissues and organs, especially the lungs, and also decreases the toxicity of NO^\cdot by acting as a reservoir for the slow release of NO^\cdot and therefore preventing the formation of peroxynitrite (Gaston et al., 1994; Meyer et al., 1994; Ohara et al., 1995; Stamler, 1994). Nitrosothiols (RSNO) are the most predominant redox form of NO^\cdot in the plasma. The most abundant thiol in plasma is albumin, which serves as a source and sink of NO^\cdot buffering the concentrations of free NO^\cdot . Alteration of the redox state of thiols by addition of NO^\cdot may also serve as a method of signaling by NO^\cdot . There is speculation that nitrosothiols participate in smooth muscle relaxation as opposed to NO^\cdot itself (Ignarro et al., 1981). Drugs used in the activation of guanylate cyclase, such as amyl nitrite and glyceryl trinitrite, induce smooth muscle relaxation, but require the presence of SH groups in the form of added thiols. The drugs then react with the cysteine group of the thiol, forming S-nitrosocysteine, which activates guanylate cyclase and increases cGMP levels to levels necessary for relaxation (Ignarro et al., 1981). The vasodilatory properties of NO^\cdot are therefore likely, at least in part, be due to the formation of the intermediate S-nitrosothiol (Ignarro et al., 1981).

Interaction of NO^\cdot with either free iron or heme and metalloproteins, is also an important biological reaction, being involved in at least 3 physiological systems including - the effector mechanisms of guanylate cyclase activation; the inhibitory action of haemoglobin in systems in which NO^\cdot is generated; and the nitrosylation of iron-sulfur centered proteins in macrophage cidal reactions (Gaston et al., 1994). NO^\cdot has also been shown to react with, and in some cases modify the function of a variety of other biological metalloproteins, such as myoglobin, cytochrome c, catalase and succinate dehydrogenase (Gaston et al., 1994; Stamler, 1997) and has been shown to

play a part in the upregulation of activity and mRNA synthesis of γ -GCS, the rate limiting enzyme in GSH synthesis (Kuo et al., 1996b).

Figure 4 gives an overview of the formation of NO \cdot , its products and some of the functions of NO \cdot .

Figure 4: Biological function of NO[•] (Robbins and Grisham, 1997).



A; NO[•] is formed by the enzyme NOS from arginine. **B;** NO[•] can stimulate guanylyl cyclase, causing an increase in cGMP formation, which in turn, relaxes smooth muscle, such as vascular smooth muscle, producing a decrease in blood pressure. This is possibly achieved by the formation of S-nitrosothiols, which release NO[•] to the iron group of the guanylyl cyclase enzyme. **C;** NO[•] can act as a neurotransmitter by stimulating NMDA receptors. **D;** NO[•] and its oxides can produce bactericidal or cytotoxic effects. They can also nitrosate proteins and thiols altering function. **E;** NO[•] can modulate inflammation, producing both anti-inflammatory effects, such as decreases in lymphocyte activation, or pro-inflammatory effects, such as increase in leukocyte migration.

4.4 NO[•] AS A MESSENGER AND TOXIN.

NO[•] can therefore act as both an oxidant and an antioxidant. In some cells NO[•] is cytotoxic and others cytostatic suggesting sensitivity to NO[•] varies from one cell to another (Vandeputte et al., 1994). Small amounts of NO[•], such as those produced by cNOS are sufficient to activate known NO[•] sensitive enzymes and participate in NO[•] signaling. Larger amounts, exceeding a critical concentration, such as those produced by iNOS during inflammation, inhibit the action of certain enzymes, such as those containing iron centres, resulting in inhibition of DNA synthesis, protein synthesis and mitochondrial enzymes (Nussier and Billiar, 1993). Table 3 shows the effects of NO[•] as both a messenger and a toxin (Schmidt and Walter, 1994).

Table 3: The effects of NO (Schmidt and Walter, 1994).

Tissue	Messenger	Toxin
Blood vessels	EDRF, antithrombic, ischemic protection, antiatherosclerotic, inhibition of smooth muscle migration and proliferation, anti-adhesive	Septic shock, inflammation, reperfusion injury, microvascular leakage, atherosclerosis
Heart	Coronary perfusion, negative inotropic, ischaemia	Myocardial “stunning”, septic shock, reperfusion
Lung	Ventilation-perfusion matching, bronchociliary motility, mucous secretion, immune defence	Immune complex-induced alveolitis, silo filler’s disease, asthma?, ARDS?
Kidney	Tubuloglomerular feedback, glomerular perfusion, renin secretion	Acute kidney failure, glomerulonephritis
CNS	Synaptogenesis, synaptic plasticity, memory formation, cerebral blood flow and ischaemia, neuroendocrine secretion, visual transduction, olfaction	Neurotoxic, proconvulsive, migraine, reperfusion
Pancreas	Endocrine/exocrine secretion	β cell destruction
Gut	Blood flow, peristalsis, exocrine secretion, mucosal protection, antimicrobial	Mutagenesis, mucosal damage
Immune system	Antimicrobial, antitumour	Anti-allograft, graft versus host disease, inflammation, septicshock, tissue damage

4.5 NO⁻ IN LUNG DISORDERS

As previously described in **Section 3**, NO⁻ has been implicated in lung disorders associated with inflammation such as asthma, ARDS, cystic fibrosis, IPF and COPD. NO⁻ is increased in the breath of asthmatics at higher levels than normal, as is iNOS

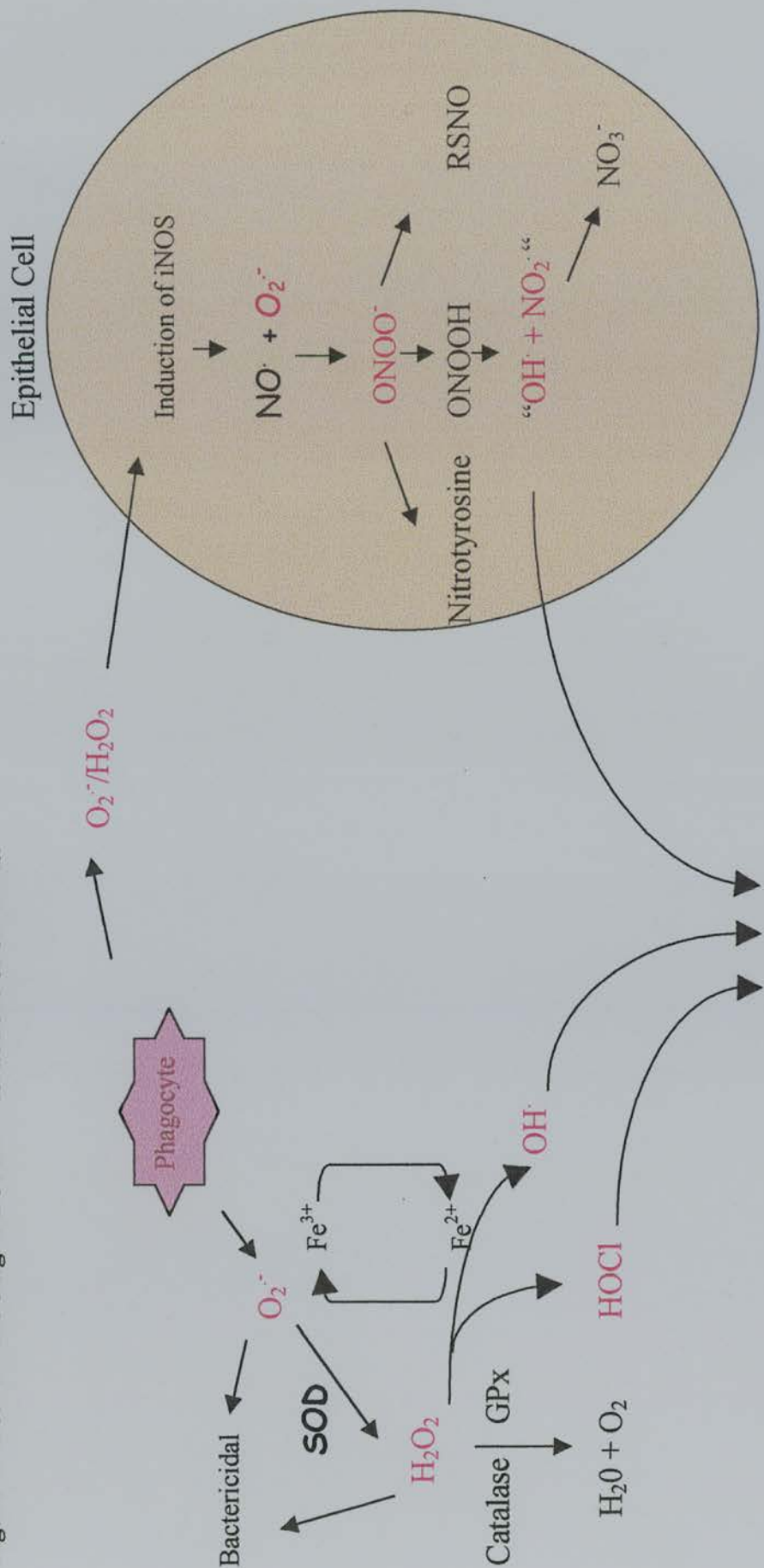
expression, (Hamid et al., 1993; Robbins et al., 1994) and this increase can be decreased by inhalation of inhibitors of iNOS (Barnes and Liew, 1995; Yates et al., 1996). NO[•] is therefore likely to have a detrimental role in asthma since inflammatory cells present in asthmatics, such as neutrophils, release oxidants during the respiratory burst, which react with NO[•] resulting in the formation of peroxynitrite. Lung biopsies of patients with ARDS, have been found to have increased levels of iNOS protein and increased levels of nitrotyrosine residues, both shown by immunohistochemistry. Increased nitrosylation of tyrosine residues in ARDS patients was shown to be associated with severity of the disease (Kooy et al., 1995; Royall.JA. and Kooy, 1997; Saleh et al., 1997). Both NO[•] and peroxynitrite are also thought to play a role in the pathophysiology of idiopathic pulmonary fibrosis (Saleh et al., 1997), cystic fibrosis (Balfour-Lynn et al., 1996), (although increased NO is not observed in the breath of patients with cystic fibrosis, possibly due to the thickness of the mucus (Balfour-Lynn et al., 1996; Saleh et al., 1997)) and chronic obstructive pulmonary disorder, induced by smoking (Flenley et al., 1986; Jeffrey, 1992).

Continuous expression of iNOS mRNA has been shown in airway epithelial cells *in vivo*, but once cells are cultured *in vitro* they lose the ability to continually express iNOS (Guo et al., 1995) and there must therefore be factors of the airway, which contribute to iNOS expression. It is likely that respiratory epithelial cells are key inflammatory cells in the airway, having a function in host defence and also playing a role in airway inflammation. iNOS mRNA can be induced in airway epithelial cells by a mixture of cytokines such as IL1- β , TNF- α , IFN- γ and LPS (Geller et al., 1993; Maier et al., 1994), however iNOS expression is present, *in vivo*, in the absence of cytokines and LPS (Nathan and Xie, 1994).

NO[•] can act as an anti-inflammatory agent in the lungs by increasing ciliary motility, thus enhancing muco-ciliary clearance and can also mediate cytotoxicity against bacteria etc (Schmidt and Walter, 1994), both of which are important features of host defence. Epithelial cells may therefore regulate an inflammatory response in the normal lung, since they have the ability to produce cytotoxic NO[•] levels. Cytotoxic

levels of NO[•] have been shown to inhibit superoxide production by neutrophils (Clancy et al., 1992) and inhibit leukocyte adhesion (Barnes, 1993; Granger and Kubes, 1996; Li et al., 1995), suggesting that epithelial cells are critical effector cells of the immune defense system in the lungs (Guo et al., 1995). Figure 5 shows the formation of NO[•], free radicals and their effect on cell damage.

Figure 5: A schematic diagram of the interactions of oxidants.



5 Glutathione (L- γ -glutamyl-L-Cysteinylglycine)

The aim of this study was to determine the role of GSH in NO \cdot induction and to examine the effect of intracellular GSH redox state on NO \cdot levels and nuclear factor kappa B (NF- κ B) activation.

GSH is a low molecular weight tri-peptide consisting of 3 amino acids; glutamine, cysteine and glycine. It was first described over 100 years ago, and its structure was established in 1935 (Morris and Bernard, 1994). The N-terminal glutamate is linked to the cysteine via a non- α peptidyl bond. Its reduction site is the reduced sulfhydryl group on the cysteine moiety and the γ -glutamyl linkage provides internal stability of GSH in the cell. GSH makes up ninety percent of all intracellular non-protein thiols. Thus, it is a key intracellular reducing agent, (Morris and Bernard, 1994) and protects cells against oxidants and xenobiotics.

The versatility of GSH includes its functions as; an antioxidant, a cysteine storage molecule, and a transport molecule. It is involved in the conjugation and detoxification of endogenous and foreign electrophilic compounds, and is necessary for the synthesis of many cellular compounds and is important in immunological modulation (Morris and Bernard, 1994). Due to its sulphhydryl group it is able to scavenge free radicals by donation of its hydrogen atom, resulting in the formation of the stable disulphide, GSSG. It can also act as a co-substrate in the reduction of H₂O₂ and other hydroperoxides by glutathione peroxidase (see 2.2). GSH is able to conjugate with a variety of compounds, via its sulphide bond or through the action of the glutathione S-transferases, a group of enzymes which are important in the detoxification of xenobiotics (Moldeus and Jernstrom, 1983). It also plays a major role in many metabolic pathways such as the reduction of disulphide linkages of proteins and DNA precursors as well as activation of enzymes and regulation of immune responses (Meister and Anderson, 1983).

GSH is therefore one of the primary cellular defences against electrophilic compounds, not only as an antioxidant but by playing a role in maintaining normal physiological function.

5.1 GSH METABOLISM

Cellular turnover of GSH occurs constantly, the oxidised form of GSH, GSSG, is removed from the cell due to its toxicity to the cell. Intracellular GSH is regulated by the externally membrane bound γ -Glutamyl transpeptidase (γ -GT), γ -Glutamylcysteine synthetase (γ -GCS) and glutathione synthase (GS). γ -GT is involved in the breakdown of extracellular GSH, into its constituent amino acids, which are transported into the cell and resynthesised into intracellular GSH by γ -GCS and GS. The enzymes are represented by the γ -Glutamyl cycle (figure 6 below) (Cantin and Begin, 1991; Meister and Anderson, 1983).

GSH synthesis requires γ -GCS, GS, ATP, Mg^{2+} and the relevant amino acids. The major organs involved in GSH synthesis are the liver and the kidney. GSH is then either used in various cellular metabolic pathways or exported out of the cell. The mechanism for exporting GSH is still not fully understood but is thought to either involve γ -GT, (Tate and Meister, 1981) or occur via a sodium transport system which may also be responsible for the uptake of intact GSH in certain epithelial cells (Burrowski et al., 1995; Hagen et al., 1988; Hagen and Jones, 1987). In the presence of an oxidative stress, GSH is oxidised to GSSG, which is highly toxic to the cells and either exported, possibly by an active transport system (Meister and Anderson, 1983) or reduced to GSH by glutathione reductase and NADPH in the GSH redox cycle (see 2.2 Figure 1).

The breakdown of both GSH and GSSG extracellularly, involves the outer membrane bound γ -GT, which catalyses the transfer of the γ -Glutamyl moiety to acceptor molecules such as amino acids (cystine, glutamine, methionine), dipeptides and other γ -Glutamyl compounds (GSH) (Tate and Meister, 1981). γ -GT is

prevalent in secretory cells such as kidney epithelial cells, the epididymus and bronchial cells, but it also occurs in alveolar type I and II cells and in alveolar macrophages (Dinsdale et al., 1992). γ -GT consists of 2 sub-units, the cysteinylglycine moiety binding to the heavy sub-unit, and the active site for transfer of the γ -Glutamyl moiety to an acceptor molecule being in the light subunit (Tate and Meister, 1981). The remaining L-cysteinylglycine from GSH is then cleaved by dipeptidases on the cell surface to release the 2 amino acids cysteine and glycine. The amino acids are then transported back into the cell. This is known as the γ -Glutamyl cycle and is represented in figure 6.

De novo synthesis of GSH intracellularly is a 2 step process:

- Glutamic acid and cysteine are combined in a step catalysed by γ -GCS. This is both the first and the rate limiting step of GSH synthesis. The active site of the enzyme can bind the γ -Glutamyl moiety of GSH and glutamate, and hence feedback inhibition of γ -GCS can occur by GSH itself and glutamate. The first step is also rate limited by the availability of cysteine. Cysteine and cystine are 2 non-essential amino acids, which are synthesised in the liver, cysteine is obtained by reduction of cystine, which is more stable in the oxidative environment of plasma. Cystine is the most active acceptor of the γ -Glutamyl moiety and therefore is taken up readily by γ -GT, it can then be reduced in the cell to cysteine and used for GSH synthesis. The availability of cystine, cysteine and a reductive environment are therefore critical to this rate-limiting step in GSH synthesis. Eighty percent of γ -GCS is inactive in the cell since it is bound to GSH. Depletion of GSH within the cell therefore results in GSH release from γ -GCS, resulting in increased GSH synthesis. mRNA for γ -GCS and γ -GT are also increased following oxidant exposure and depletion of GSH (Liu et al., 1996).
- The addition of glycine to γ -Glutamylcysteine is achieved by the enzyme GSH synthetase (GS). This is not a rate limiting step, and probably plays a less important role than γ -GCS in an oxidant response.

5.2 GSH AND THE LUNGS

As already discussed in the section on antioxidants, the ELF of the normal lung contains high concentrations of GSH (Cantin et al., 1987) since the lung is exposed to potentially toxic oxidation from various sources. GSH levels in the ELF of a normal individual are approximately 100 times greater than plasma levels, and it is thought that alveolar macrophages play a major part in releasing GSH since they

contain high levels of GSH (9 times more than alveolar type II cells) and they have an active γ -GT cycle (Cantin and Begin, 1991; Horton et al., 1987; Liu et al., 1996). GSH is able to protect the cell membrane against oxidative damage by maintaining SH groups and reducing compounds in the immediate environment of the cell and can prevent the oxidation of antiproteases (Morris and Bernard, 1994).

GSH is likely to be critically involved in the negative control of pro-inflammatory cytokines and NO⁻ production (Galter et al., 1997; Hayashi et al., 1993; Liu et al., 1996; Staal et al., 1990). An increased oxidant burden, which results in alterations in the GSH redox state of the cell, results in the activation of NF- κ B, a transcription factor which switches on pro-inflammatory cytokines such as TNF- α and IL8 and NO⁻ production (Fujihara et al., 1994; Jany et al., 1995; Mellits et al., 1993; Newton et al., 1997; Sen and Packer, 1996).

6. Nuclear Factor-KappaB (NF- κ B)

NF- κ B has been shown to induce mRNA for iNOS in many cell types and multiple putative NF- κ B sites in the promoter region of iNOS have been shown to be critical for iNOS induction in response to stimuli such as cytokines (Chartrain et al., 1994; Natoli et al., 1997). Activation of NF- κ B is thought to be redox regulated, having an oxidant step in its activation that is inhibitable by thiols (Flohe et al., 1997; Sen and Packer, 1996). NF- κ B nuclear binding has been studied in this thesis in response to various stimuli such as cytokines and oxidants, and the effects of thiols on NF- κ B induction have also been investigated. This section covers the structure and activation of NF- κ B and discusses the controversy surrounding NF- κ B activation. It also covers the role of NF- κ B in the pro-inflammatory processes in lung inflammatory disease.

Acute and chronic alveolar and bronchial inflammation is a central feature of the pathology of lung disorders such as asthma, COPD, ARDS and IPF. All of these disorders lead to the recruitment to, and activation of immune and inflammatory cells

in the lungs. These inflammatory cells release cytokines, oxidants and other inflammatory mediators. Epithelial cells also secrete pro-inflammatory mediators, including NO[•], oxidants and cytokines, which contribute to a chronic inflammatory response which can cause lung injury (Rahman and MacNee, 1998). NF- κ B is a major regulator of pathogen and inflammatory cytokine-inducible gene regulation (Baeuerle and Baichwal, 1997; Flohe et al., 1997; Rahman and MacNee, 1998)

NF- κ B binding sites serve as inducible transcriptional regulatory elements that respond to immunological stimuli such as TNF- α , LPS and IL1- β . Inducers of NF- κ B, however, are not exclusively immunological, but also include an array of substrates such as oxidants, growth factors, viral infection and UV light. NF- κ B is associated with an I κ B subunit in the cytoplasm, release of which causes rapid translocation of NF- κ B into the nucleus, activating genes within minutes. There is increasing evidence to support the occurrence of interactions between NF- κ B and other transcription factors resulting in the regulation of gene expression in a selective manner (Hecker et al., 1996a; Lincoln et al., 1996; Salkowski et al., 1996). Synergy with other transcription factors may determine cell type specific expression of genes, an example being interferon regulatory factor-1 (IRF-1), which is inducible by IFN- γ treatment and physically interacts with NF- κ B subunits (Baldwin, 1996; Salkowski et al., 1996). Activation of IRF-1 by IFN- γ , although not essential, is thought to be required for optimal expression of the iNOS gene (Salkowski et al., 1996). NF- κ B activation is therefore necessary, but not necessarily sufficient for inducible expression of particular genes. A great degree of specificity can therefore be achieved by the synergistic interactions of NF- κ B with other factors in gene regulation (Baeuerle and Baichwal, 1997; Hecker et al., 1996a).

6.1 STRUCTURE

The NF- κ B subunits and I κ B proteins belong to larger protein families. Among the NF- κ B subunits, 5 rel proteins have been recognised and characterised, and all

contain the Rel homology domain (RHD), a highly conserved protein involved in immune regulation in insects (Warner et al., 1995). The 5 rel proteins are; cRel, RelA (p65), relB, NF- κ B 1 and NF- κ B 2. The rel homology domain functions in DNA binding, dimerization and interactions with I κ B. Active forms of NF- κ B include the p50/p65 (relA) heterodimer and the p65/p65 (RelA/cRel) homodimer. Different dimers are able to recognise different DNA consensus targets, thereby increasing the ability of NF- κ B subunits to differentially regulate gene expression (Baldwin, 1996). The p50/p65 heterodimer plays a critical role as a potent transcriptional activator that is most frequently found in inducible complexes of various immune and non-immune cell types. In the nucleus the p50 subunit facilitates binding to DNA whereas the p65 subunit is required for transactivation. A p50 homodimer also exists and this is able to bind DNA and inhibit transcription (Baldwin, 1996; Rahman and MacNee, 1998).

6.2 THE INHIBITORY SUBUNIT I κ B

There are several forms of I κ B including I κ B- α and I κ B- β , and their purpose is to inactivate NF- κ B by masking the nuclear binding sequence. A feature of I κ B is the presence of ankyrin repeats, and since both NF- κ B 1 and 2 contain these ankyrin repeats it is assumed that they contain an intramolecular I κ B. NF- κ B 1 and 2 are thought to be precursor molecules for the p50 and p52, subunits of NF- κ B, which bind DNA. I κ B- α , the most studied inhibitor of NF- κ B activation, is often associated with the cRel and RelA dimers. The ankyrin repeats, present in all I κ B subunits, are required for binding to the Rel homology domain of NF- κ B subunits. The I κ B α protein contains 3 domains, the N domain, C domain and an intermediate domain. Experiments to remove or inactivate the N and C domain did not inhibit I κ B α interaction with NF- κ B. However on removal of the C terminal, NF- κ B was able to bind to DNA indicating that the C terminal is involved in masking of the DNA binding site on NF- κ B. The intermediate domain contains the ankyrin repeats and mutations in this region prevents I κ B binding to NF- κ B. I κ B β is similar to I κ B α but is targeted by different stimuli (Baldwin, 1996).

6.3 ACTIVATION OF NF- κ B

NF- κ B is activated by the phosphorylation and ubiquitination of I κ B, resulting in degradation of the subunit. This can be demonstrated by the use of protease inhibitors which block activation of NF- κ B (Mellits et al., 1993). Protease inhibitors inhibit the processing and degradation of I κ B but phosphorylation still occurs (Mellits et al., 1993), suggesting that a further step, the ubiquitination step, is required for the degradation of I κ B. The I κ B gene is activated by NF- κ B itself, and is regenerated one hour after NF- κ B activation, re-entering the cytosol and inactivating NF- κ B. I κ B is therefore important in repression after induction. Phosphorylation of I κ B is thought to be achieved by a single kinase since mutations of serine residues on I κ B inhibit both TNF- α and LPS activation of NF- κ B (Baldwin, 1996). This kinase has recently been identified as the NF- κ B inducing kinase (NIK) (Regnier et al., 1997). However, it is likely that this kinase may itself be activated by multiple pathways (Baeuerle and Baichwal, 1997).

Secondary messengers are also thought to be involved in NF- κ B activation. These include TNF- α activated ceramide, (which is thought to activate kinases which in turn phosphorylate I κ B), and ROS, (since H₂O₂ has been shown to increase NF- κ B activation) (Kretz-Remy et al., 1996; Schreck et al., 1991). TNF- α itself, results in increased ROS release from the mitochondria, which is dismutated by SOD into H₂O₂. There is conflicting evidence that ROS are important in the activation of NF- κ B, (Brennan and O'Neill, 1994; Flohe et al., 1997; Sen and Packer, 1996) and it is likely that different systems occur in different cell types.

6.4 REDOX REGULATION OF NF- κ B.

NF- κ B can thus be activated by H₂O₂, TNF- α , and other cytokines (Fujihara et al., 1994; Jany et al., 1995; Mellits et al., 1993; Newton et al., 1997) and there appears to be a common link in the activation of NF- κ B, by ROS which can be blocked by antioxidants such as NAC (Blackwell et al., 1996; Hecker et al., 1996a; Schenk et

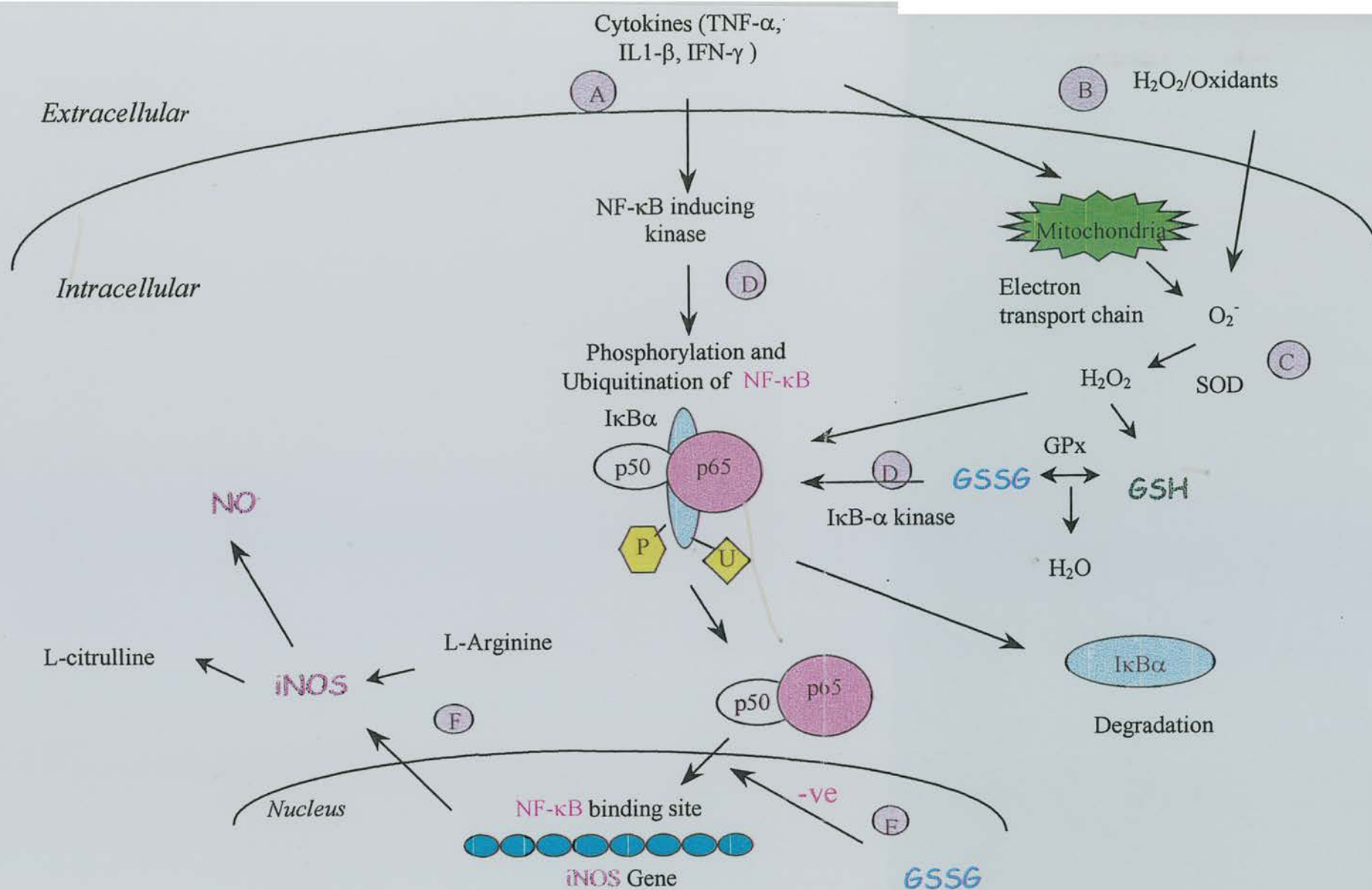
al., 1994; Schreck et al., 1991). There are, however, several reports showing that oxidants are not necessary for NF- κ B activation, particularly by TNF- α and IL1- β (Brennan and O'Neill, 1994). Moreover, antioxidants are also able to activate NF- κ B (Kumuda et al., 1995; Toledano and Leonard, 1991). The involvement of ROS in NF- κ B activation has been assumed to be due to an oxidant step in the cascade leading to NF- κ B activation, possibly by modification of a protein. There are several reports however, suggesting that oxidants are not directly involved in NF- κ B, but that the redox status of the cell is important, in particular, GSH, GSSG and thioredoxin (Trx) (Galter et al., 1997; Hayashi et al., 1993; Staal et al., 1990). A potent inducer of NF- κ B activation is tertradecanoyl-phorbol-13-acetate (TPA), which has been shown to increase GSSG levels, altering the GSH:GSSG ratio (Droge et al., 1994a; Fidelus, 1998). NF- κ B activation can be inhibited by BSO, a compound that inhibits GSH synthesis (Meister and Anderson, 1983), and augmented by BCNU, a compound that inhibits GR and reduction of GSSG (Galter et al., 1997), thereby increasing GSSG. GSSG has an important role in protein disulphide formation and protein folding, and may be necessary in forming a disulphide bond in the NF- κ B cascade (Droge et al., 1994). It has also been shown that GSSG is necessary for both induction and nuclear translocation, but excess GSSG inhibits DNA binding (Droge et al., 1994; Galter et al., 1997). Evidence thus suggests that the level of GSSG in the cell is critical in both NF- κ B activation and inhibition (Droge et al., 1994a). Oxidative conditions have been shown to inhibit DNA binding of NF- κ B (Galter et al., 1997). NF- κ B has a sequence motif containing cysteine and arginine in the DNA binding region (Matthews et al., 1992). The cysteine residue is highly reactive and susceptible to oxidation, and binding can be inhibited by modification of the cysteine residue, or enhanced by reducing agents.

Optimal activity of NF- κ B may be sensitive to an oxidant/antioxidant balance in the cell, and may also depend on optimal levels of GSSG. Low GSSG levels prevent NF- κ B activation, and high GSSG levels inhibit NF- κ B binding to DNA (Droge et al., 1994a; Galter et al., 1997). Alternate pathways may also exist for some cytokines such as TNF- α and IFN- γ , which have been shown to activate NF- κ B in the presence

of antioxidants (Brennan and O'Neill, 1994). It is likely, however that the reductive state of the nucleus is vital in the binding of NF- κ B to DNA due to the presence of cysteine residues in the binding site (Matthews et al., 1992).

Figure 7 shows a schematic view of the systems possibly involved in NO \cdot induction, including an oxidant/antioxidant pathway for NF- κ B activation as well as an alternative pathway independent of oxidants.

Figure 7: A schematic view of NO[•] induction via NF-κB activation.



A: Pro-inflammatory cytokines such as TNF-α bind to receptors on the cell surface, causing protein kinase phosphorylation and superoxide anion release from the mitochondria. B: Addition of H₂O₂ to the cell mimics the effect of superoxide anion release from the mitochondria. C: Superoxide anions are converted to H₂O₂ (by SOD), which is broken down to water by glutathione peroxidase (GPx) and GSH. D: H₂O₂, GSSG and cytokines may all have effects on phosphorylation of NF-κB either directly or by phosphorylation of protein kinases, GSSG in particular may be involved in ubiquitination. This results in degradation of the IκB subunit and activation and translocation of NF-κB to the nucleus. E: A reductive environment is required in the nucleus for NF-κB binding to DNA. Binding may be prevented by too high concentrations of GSSG. F: NF-κB binds to DNA, switching on transcription of the gene for iNOS. NO[•] is formed by the enzyme iNOS and L-arginine.

7 Hypothesis

The redox status of the cell, particularly that of GSH is critical to the induction of NF- κ B activation by cytokines and oxidants, which subsequently involves the induction of iNOS and NO \cdot production. Increasing intracellular GSH by exogenous thiol compounds will thus prevent NO \cdot induction.

8 Aims

The studies herein were designed to induce iNOS and NO \cdot production through the activation of NF- κ B by pro-inflammatory cytokines and oxidants (H₂O₂), in 2 cell lines, *in vitro*, and to determine the role of intracellular GSH in this induction.

8.1 SPECIFIC OBJECTIVES:

- To measure nitrite release, as an indication of NO \cdot production and iNOS mRNA levels upon stimulation of cells with a mixture of pro-inflammatory cytokines, LPS and H₂O₂.
- To determine the effect of NO \cdot induction on GSH levels of the cell.
- To determine the effect of depleting GSH levels with BSO, on NO \cdot induction
- To increase intracellular GSH levels using four exogenous thiol compounds NAC, NAL, GSH, and GSHMEE.
- To determine the effect of increased intracellular GSH levels on the induction of NO \cdot and iNOS mRNA, by cytokines and H₂O₂.
- To determine the effect of cytokines and H₂O₂ on NF- κ B activation
- To determine the effect of modulating GSH levels, by BSO and thiol compounds, on cytokine and H₂O₂ activation of NF- κ B.

Chapter 2 Materials and Methods.

2.1 MATERIALS.

See Appendix 1.

2.2 METHODS.

2.2.1 CELL CULTURE TECHNIQUES.

Two cell lines representative of bronchial and alveolar cells were used throughout this project: a human type II alveolar-like cell line, A549, and a human bronchial epithelial cell line, 16HBE140-.

The A549 cell line has been recently proposed as a typical lower respiratory airway cell and is derived from a single patient with a pulmonary adenocarcinoma (Lieber et al, 1976). The cells were obtained from the American Type for Cell Culture, (ATCC).

Dr. D.Greunert of the University of California kindly donated the bronchial epithelial cell line, 16HBE140-. These cells were clone 16 derived from bronchial tissue of a healthy male. The nomenclature 0- indicates they were transformed by the SV40 virus and the 14 stands for the 14th sample. These cells have been shown to possess cilia (Cozens et al., 1994) and express the chloride channel gene CFTR, which has been shown to be defective in cystic fibrosis.

Both cells were grown in monolayers in 160mm³ flasks. Once they reached 95% confluency they were passaged using phosphate buffered saline (PBS), trypsin/EDTA and complete medium, all at 37°C. Confluency throughout this project is defined in terms of percentage of plastic (wells/flasks) covered by cells, and is assessed visually, although cells were also counted. Cells were normally treated at 70% confluency for a maximum of 24 hours at which point cells had



reached 95-99% confluency, defined as greater than 95% of the culture dish covered by the cells. A549 cells were grown in Dulbecco's modified eagles medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 100mM penicillin (P) and 1000u/ml of streptomycin (S) (known as the penicillin/streptomycin mixture (P/S)) and 2mM l-glutamine (L-G) hence forth referred to as A549 complete medium. The 16HBE cells were grown, in contrast to the A549 cells, in minimum essential medium (MEM), supplemented with 10% FBS, P/S, L-G and 1% non-essential amino acid mixture (AA) and is henceforth be referred to as 16HBE complete medium.

To passage the cells, medium was aspirated from the flasks and the cell monolayer rinsed in warm sterile PBS. Cells were detached from the flask by the addition of 7ml of warm trypsin/EDTA, A549 cells requiring 5 minutes incubation at 37°C and 16HBE cells requiring 10 minutes incubation at 37°C. Once detached 20ml of complete medium was added to the flask to inactivate the trypsin, and the resultant cell suspension was transferred to a sterile 50ml falcon tube. Trypsin/EDTA was removed by centrifugation at 1000rpm for 5 minutes at room temperature (RT) and the cell pellet was resuspended in the volume of complete medium necessary for the next stage. Generally cells were resuspended in; 20ml of complete medium for propagation, 5ml of the suspension being transferred to a sterile flask along with 20ml of complete medium, or 24ml of complete medium for experimental purposes, 1ml of suspension being transferred to a well in a 6 well plate along with 2ml of complete medium. Cell cultures were maintained at 37°C, 5% CO₂. Flasks for propagation were left until cells required further passaging with a change of medium every 2 days. Cells for experimental use were incubated until they reached 70% confluency (approximately 1.4×10^6 cells/well of a 6 well plate), usually the following day, and were then treated.

All cell culture work was performed in a uni-directional laminar flow Class II safety cabinet. The surface of the cabinet was sprayed with isopropanol as was all bottle caps and flasks caps prior to opening inside the cabinet. The appropriate protective clothing (lab coat and disposable gloves) were worn on all occasions. All waterbaths

and incubator humidifiers were cleaned on a weekly basis to reduce the risk of bacterial contamination. On rare occasions when infection occurred, cells and medium were destroyed and cells from frozen stock were seeded in new medium. All cells were regularly checked for mycoplasma contamination and the cells used were mycoplasma free.

Vials of both cell types were frozen down at regular intervals to ensure a sufficient reserve in case of infection as mentioned above. This process involves removal of cells from the flasks using trypsin/EDTA as described above. However cells were resuspended in 1ml of freezing solution, (80% complete medium plus an extra 10% FBS and 10% dimethylsulfoxide (DMSO)), and transferred to a cryogenic sterile 2ml vial. DMSO inhibits the formation of ice crystals within the cells during the freezing process since ice crystals occupy more space than water and would therefore lead to cell disruption. However, DMSO is toxic to cells at room temperature and it is therefore important to freeze the cells immediately. The vials were wrapped in tissue paper and placed at -80°C for 48 hours and then transferred to a liquid nitrogen storage unit (-130°C) for long term storage. The process of freezing down slowly using 2 temperature stages also helps minimise the formation of ice crystals (Morgan and Darling, 1996).

Likewise when cells were required from stock they were thawed out rapidly by placing them at 37°C until they had thawed out. They were then aseptically transferred to 10ml of warm sterile complete medium, washed by centrifugation at 1000rpm, 25°C, for 5 minutes and the pellet resuspended in 10ml of fresh complete medium before seeding into a 160mm³ flask. Once the cells had adhered, the medium was replaced to remove any remaining traces of DMSO.

2.2.2 EXPERIMENTAL TREATMENT OF CELLS

All cells were treated in phenol red free medium supplemented with 2% bovine serum albumin (BSA), P/S, L-glutamine with 1% non-essential amino acids for the

16HBE cells, hence forth referred to as 2% medium. Cells were washed twice in PBS when 70% confluent (approximately 1.4×10^6 cells per well of a 6 well plate), treated with bolus additions of appropriate treatment at the correct concentrations and incubated for the appropriate time at 37°C, 5% CO₂.

Concentrations of treatments and dilutions of compounds used throughout were as follows:

Cytomix (TNF- α 10ng/ml, IFN- γ 10ng/ml, IL-1 β 10ng/ml), cytokines were dissolved in PBS containing 1% BSA and diluted to a storage concentration of 10 μ g/ml and stored at -70°C. Cytokines were diluted to 10ng/ml in 2% medium for experimental use.

LPS (from E.Coli serotype OIII:B4, γ -irradiated) was diluted to 1mg/ml in PBS and stored at -20°C. LPS was diluted to 1 μ g/ml in 2% medium and sonicated for 10 minutes before treatment of cells.

H₂O₂ (1 μ M), BSO (50 μ M), L-NMMA (50 μ M), NAC (5mM), NAL (5mM), GSH (5mM) and GSHMEE (5mM) were all diluted in 2% medium for experimental use.

Rotenone was dissolved in chloroform at 0.1 μ g/ml.

2.2.3 ASSAYS.

2.2.3.1 PREPARATION OF CELLS PRIOR TO ASSAYS.

Various assays were performed on both cell medium and cell lysates. Following treatments and incubation, medium was removed from the cells and stored on ice for nitrite measurement. Cells were subsequently removed from the wells as described above by washing in warm PBS and incubation at 37°C for 5-10 minutes in 0.5ml of trypsin/EDTA. Cell suspensions were aspirated from the wells, placed in 2ml

Eppendorfs with 1ml of warm complete medium to wash the cells and centrifuged at 1000rpm for 5 minutes at room temperature. The supernatant was removed and cells were resuspended in 1ml PBS. An aliquot (20µl) of the cell suspension was removed to determine cell numbers and to check viability, and the remaining cells were re-spun at 1000rpm, 37°C for 5 minutes. The supernatant was removed and cells were homogenised in 1ml of 0.6% Sulfosalicylic acid (SSA), 0.1% triton in PBS using a mini-teflon. Samples were then spun at 2500rpm for 10 minutes at 4°C and kept on ice or stored at -20°C for GSH measurements.

2.2.3.2 CELL COUNTS AND CELL VIABILITY.

Cells were counted using a standard haemocytometer and trypan blue. Trypan blue exclusion was used as an indication of cell viability.

2.2.3.3 NITRIC OXIDE (NO[•]) ASSAY.

Nitric oxide (NO[•]) released into the medium rapidly converts to nitrite (NO₂⁻). Nitrite was measured using the Griess reagent (Green et al., 1982) which involves the diazotization of sulfanilimide and subsequent coupling to the chromophore N-(1-naphthyl)-ethanediamine. This results in a colour change which was measured spectrophotometrically at 550nm on a Dynatech 3000 plate reader. In order to observe the colour change, phenol red free medium was used to measure nitrite, 100µl of cell medium was placed into a 96 well flat bottom plate and 100µl of Griess reagent (1% sulfanilomide, 0.1% N-(1-naphthyl)-ethanediamine and 2.5% H₃PO₄) was added to the plate which was incubated at room temperature for 10 minutes. A standard was established using sodium nitrite, diluted in distilled water (dH₂O) in the range of 0.098µM to 50µM. The results were expressed per million cells.

2.2.3.4 REDUCED GLUTATHIONE (GSH) ASSAY.

GSH was measured using the Tietze assay (Tietze, 1969), which involves the glutathione redox cycle i.e. oxidised GSH (GSSG) is reduced by glutathione reductase (GR) and NADPH. To detect GSH the compound 5,5'-dithio-bis-(2-Nitrobenzoic acid) (DTNB) is used which binds to thiol groups and forms a complex with an absorbance at 412nm. The rate of formation of the DTNB-GSH complex is directly dependent on the concentration of GSH present in the sample. This assay measures total GSH and does not distinguish between oxidised (GSSG) and reduced glutathione (GSH) since GSSG is recycled to GSH.

For the assay 680-690µl phosphate buffer (PB) (pH 7.5, containing 5mM of EDTA), 100µl GR (10units/ml PB), 100µl DTNB (5mM in PB), and 10-20µl of sample are placed into a 1ml cuvette and left at room temperature for 1 minute. One hundred µl of NADPH (2.4mM in PB) was added and the rate of the GSH-DTNB complex formation followed at 412nm for 1 minute. A standard curve was established using GSH, made up in 0.6% SSA and 0.01% triton-X100, in the range of 0.02nmoles to 2.6nmoles and results were expressed per million cells.

2.2.3.5 OXIDISED GSH (GSSG).

GSSG was also measured using the Tietze method (Tietze, 1969) with an additional step that first removed GSH (Griffith, 1980). Two hundred µl of sample was transferred into an eppendorf to which 4µl of 2-vinylpyridine (2-VP) was added, the mixture vortexed and incubated for 1 hour at RT. 2-VP binds specifically to reduced GSH, thus ensuring that only GSSG will be measured subsequently in the Tietze assay. After 1 hour 6µl of 1:3 Triethanolamine (TEA) is added which inhibits the action of 2-VP. At this point the pH of the samples is important in the assay, but due to the small volumes used it was not feasible to measure the pH. There were therefore variations within the pH levels of different experiments resulting in variation between experiments, and therefore statistical analysis was not possible. However in the results shown, the trends for each experiment were the same,

allowing the data to be meaned and therefore provide some evidence for changes following certain treatments. One hundred μl of the sample was added to 100 μl GR, 100 μl DNTB and 600 μl PB as for the measurement of total GSH and incubated at RT for 1 minute. One hundred μl of NADPH was added and the absorbance read at 412nm for 2 minutes. A standard curve was established using GSSG in the range of 0.02nmols to 2.6nmols, which was treated in the same manner as the samples.

2.2.3.6 MODIFICATION OF TIETZE METHOD.

The Tietze method was modified during the project to enable the measurement of GSH/GSSG to be carried out on a plate reader. The same concentrations of reagents were used but scaled down by 1/5th of the original volume. However as a 412nm filter was not available for the plate reader a 410nm filter was used. Comparisons of the two wavelengths were carried out on the spectrophotometer (figure 1) and no significant difference between either wavelength was observed between control and treated cells. This method has since been published, (Vandeputte et al., 1994) although enzyme concentrations were different to the ones used in our assay. The difference observed between control cells and cytomix treated cells in figure 1 is explained in further detail in chapter 4.

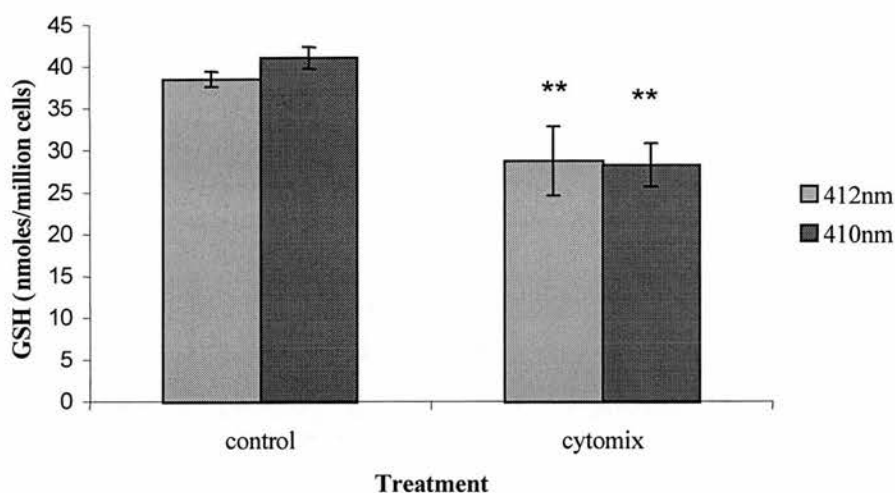


Fig 1: The effect of cytomix on GSH levels in A549 cells. There is a significant difference between control cells and cytomix treated cells at both 410 and 412nm. There is no significant difference between the two wavelengths in either control cells or cytomix treated cells. Histograms represent means and bars represent SEMs of 3 samples measured in duplicate. (** $p < 0.01$ compared to relevant control)

2.2.3.7 TOTAL PROTEIN ASSAY.

Total protein was measured using bicinchonic acid which forms a colour complex with proteins at 570nm (Smith et al., 1985). In this assay 10 μ l of sample is incubated with 200 μ l of dilute bicinochoic acid (1:50) in a 96 well plate for 30 minutes at 37°C. Absorbance is measured at 562nm using a standard of pure bovine serum albumin in the range of 0.1-0.6 mg/ml. Pierce and Warriner, Chester, UK provided a protein assay kit containing both the standard and acid.

2.2.4 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION.

2.2.4.1 RNA EXTRACTION.

Cells were passaged as previously described and seeded in 100mm petri dishes at a density of 4×10^6 cells per dish. The cells were treated when 70% confluent for the specified time. Following treatment the medium was removed and discarded. One ml of TRIzol reagent was added to the plate for 5 minutes at room temperature to lyse the cells. TRIzol reagent is used since it maintains the integrity of RNA whilst disrupting cells and dissolving cellular components. Cell lysates were removed and transferred to sterile, RNase free, 2ml Eppendorfs. Phase separation of RNA, DNA and protein was achieved by addition of 0.2ml of chloroform followed by vortexing for 30 seconds and incubation at room temperature for 2 minutes. Chloroform separates the sample into an aqueous phase and an organic phase, with RNA remaining in the upper aqueous phase. Samples were centrifuged at 12,000rpm for 15 minutes at 4°C and the aqueous phase was transferred to a sterile, RNase free, 2ml eppendorf. 0.5ml of isopropanol was added to precipitate the RNA from the aqueous phase extract, samples were incubated on ice for 10 minutes and centrifuged at 12,000rpm for a further 10 minutes at 4°C. The supernatant was discarded and the RNA pellet washed in 75% ethanol (200µl). Samples were vortexed and spun at 7,500rpm for 5 minutes at 4°C. The ethanol was removed and the cell pellets allowed to air dry on the bench for 10 minutes. Pellets were dissolved in 50µl dH₂O at 55°C for 5 minutes. Samples were either used for cDNA preparation immediately or stored at -70°C.

2.2.4.2 CDNA PREPARATION.

The total RNA concentration of the samples was determined spectrophotometrically at 260nm in a quartz cuvette. 1µl of sample was diluted into 1ml dH₂O and the RNA concentration determined. RNA/protein ratio was also determined to give an

indication of RNA purity. Table 4 explains which components are necessary for the synthesis of first strand cDNA.

Table 4 The Components for First Strand cDNA

Components	Final concentration	Final Volumes
RNA	5µg	10µl (approximately)
5x First strand buffer	1x	5µl
DTT (100mM)	10mM	2.5µl
Oligo dT (100µg/ml)	10µg/ml	2.5µl
DNTPs (10mM)	0.5mM	1.25µl
RNase inhibitor (250 units)	10 units	1µl
M-MLV Reverse Transcriptase (5000 units)	200 units	1µl
DH ₂ O	-	1.75µl (approximately)

This cDNA mixture was incubated on a PCR block for 60 minutes at 37°C after which reverse transcriptase was heat inactivated at 95°C for 5 minutes.

2.2.4.3 PCR.

For detection of mRNA using the reverse transcriptase method, 5µl of cDNA stock was added to 50µl of PCR mixture in a 0.5ml Eppendorf. PCR mixture consists of PCR buffer (50mM KCl, 10mM TrisCl pH 8.4, 2.5mM MgCl₂, 0.2mM dNTPS) and 1µM each of the appropriate primers for the particular mRNA required. Primers were used for both iNOS and GAPDH mRNA. The latter as a housekeeping gene to ensure that the PCR reaction is working and also to compare with the iNOS mRNA. Primer pairs for GAPDH were:

5' CCA CCC ATG GCA AAT TCC ATG GCA 3'
5' TCT AGA CGG CAG GTC AGG TCC ACC 3'

Primer pairs for iNOS were:

5' GTG AGG ATC AAA AAC TGG GG 3'

5' ACC TGC AGG TTG GAC CAC 3'

2 drops of mineral oil was added to the Eppendorfs to ensure evaporation did not occur during the PCR cycles. The samples were placed in the PCR thermal cycler and heated to 98°C for 10 minutes. 1Unit of Taq DNA Polymerase was added after the first 5 minutes, a feature known as 'Hot Start'. 'Hot Start' ensures that there is no non-specific binding of the primers to the cDNA as they may anneal at room temperature since Taq polymerase works at both room temperature and higher temperatures. The samples were then run on the correct program for either iNOS or GAPDH. Amplification of iNOS consists of 35 cycles of 94°C for 2 minutes, 58°C for 2 minutes, 72°C for 2 minutes and finally 1 cycle of 72°C for 10 minutes. Amplification of GAPDH consists of 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and finally one cycle of 72°C for 5 minutes. A positive control for iNOS was provided by Dr Serpil Erzurum of the Cleveland Clinic Foundation. The amplified products were resolved by agarose gel electrophoresis in a 1.6% agarose in 0.5% TBE buffer (0.45M TrisBorate and 0.01M EDTA(Na), visualised by ethidium bromide and photographed.

2.2.4.4 ELECTROPHORETIC MOBILITY GEL SHIFT ASSAY.

This assay detects DNA binding proteins such as sequence specific transcription factors. The principal of the assay is based on the migration of protein-DNA complexes through a denaturing polyacrylamide gel in which unbound DNA fragments or double stranded oligonucleotides migrate at different rates to protein-DNA complexes. The complex can be observed by the use of ³²P end labeled DNA fragments, which contain the putative protein-binding site. A gel shift assay core system was obtained from Promega for this assay.

2.2.4.5 NUCLEAR PROTEIN EXTRACTION (Andrew and Faller, 1991).

Nuclear protein extraction involves the use of 3 separate buffers;

Buffer A contains: 10mM HEPES pH7, 10mM KCl, 2mM MgCl₂, 1mM DTT, 0.1mM EDTA, 0.2mM NaF, 0.2mM sodium orthovanadate, 0.8mM PMSF and 0.3mg/ml leupeptin.

Buffer B consists of 10% Noidet P 40 diluted in distilled water.

Buffer C contains: 50mM HEPES pH7.5, 50mM KCl, 300mM NaCl, 0.1mM EDTA, 1mM DTT, 10% glycerol, 0.2mM NaF, 0.2mM sodium orthovanadate and 0.6mM PMSF.

In both buffers A and C, anti-proteases (PMSF and leupeptin) were added at the last minute.

Cells were grown in 100mm³ petri dishes until 70% confluent and treated for the appropriate time. The cells were washed in PBS, scraped into 1ml PBS, transferred to a 2ml Eppendorf and centrifuged at 1400rpm for 1 minute at 4°C. The supernatant was discarded, the pellet resuspended in 0.4ml of buffer A and the samples were incubated on ice for 15 minutes. Buffer B (25µl) was added, the samples vortexed and centrifuged at 14000rpm for 1 minute at 4°C. The supernatant was again discarded, the pellet resuspended in 50µl buffer C and samples incubated on a rotating table for 20 minutes at 4°C. The samples were centrifuged at 14000rpm for 5 minutes at 4°C, the supernatant was removed, total protein concentration was determined in the supernatant (using the protein assay described above) and the supernatant samples were stored at -20°C.

2.2.4.6 LABELLING OF THE CONSENSUS OLIGONUCLEOTIDE FOR NF-κB

The NF-κB oligonucleotide (supplied by Promega) was labeled using [$\gamma^{32}\text{P}$] ATP. Two µl of NF-κB oligonucleotide sequence (at 1.75pmol/ml) was incubated with 1µl 10xT4 polynucleotide kinase buffer, 1µl [$\gamma^{32}\text{P}$] ATP (3000Ci/ml), 5µl dH₂O and 1µl T4 polynucleotide kinase (5-10U/ml) for 10 minutes at room temperature. The

reaction was terminated by the addition of 1µl 0.5M EDTA, the reaction mixture was made up to 100µl with TE buffer (10mM Tris pH 8, 1mM EDTA, pH 8) and the labeled oligonucleotide could be stored at -20°C for up to 2 weeks.

2.2.4.7 SAMPLE PREPARATIONS

Nuclear extract proteins were diluted to 1µg/ml with dH₂O and 2µg was incubated in 5µl dH₂O, 2µl gel shift binding 5x buffer (supplemented with 0.25mg/ml sheared salmon sperm DNA) for 5 minutes at room temperature. A positive control from HeLa nuclear extract was used (provided by Promega) and a negative control tube was set up with dH₂O. One µl of the labeled oligo NF-κB was added to the samples followed by a 20 minute incubation at room temperature. The reaction was terminated by the addition of 1µl 10x gel loading buffer (20mM HEPES, 0.1% bromophenol blue, 20% glycerol). Samples were then run on a 5% acrylamide gel which is prepared by using 10ml of 30% (w/v) acrylamide/bis solution (37.5:1) diluted with 1x TBE, 50µl of N',N',N',N', Tetramethylethylenediamine (TEMED) and 50µl of 25% ammonium persulphate (APS). Samples were loaded once the gel had set and run at 100 volts for 2 hours. Gels were placed onto Whatman filter paper, vacuum dried for 30 minutes and autoradiographed.

2.2.5 IMMUNOPEROXIDASE (ABC) CYTOKERATIN STAINING OF CELLS FOR EPITHELIAL MARKERS.

Cells were seeded at 0.5×10^6 cells/ml onto sterile cover slips which were placed in petri dishes and cultured until they reached 70% confluency. Medium was removed and cells were washed in warm PBS, and then fixed using 3% paraformaldehyde (diluted in PBS without calcium/magnesium) for 4 minutes. Coverslips were then rinsed 3 times in PBS and 1mM EDTA/PBS was added to the petri dish. Cells were permeabilised by placing them in a microwave for 30 seconds (Morgan and Darling,

1996). Petri dishes were then placed in a humid chamber, cells face up, and incubated with 200µl of the Dako monoclonal mouse anti-human cytokeratin antibody at a dilution of 1 in 50 in EDTA/PBS and left for 1 hour at room temperature. Coverslips were washed with EDTA/PBS 3 times and subsequently incubated with 200µl of the secondary biotinylated antibody, rabbit anti-mouse (RAM) at a dilution of 1 in 300 in EDTA/PBS for 45 minutes in a humid container. Coverslips were rinsed in PBS, incubated with 200µl of the avidin biotinylated complex (ABC), containing horseradish peroxidase, (which is supplied in kit form from Dako), and incubated for 45 minutes. Cells were developed in diaminobenzidine (DAB) for 7 minutes, and rinsed with PBS. The nuclei were stained by addition of 10ml of nuclear fast red (filtered) to the coverslips for 5 minutes followed by rinsing in distilled water.

2.2.6 STATISTICAL ANALYSIS

All experiments were carried out a minimum of 3 times, each experiment was performed in triplicate and all assay measurements performed in duplicate. Data was analysed for skewness followed by a one way analysis of variance (ANOVA), and subsequently by Tukeys T test for unpaired samples. The histograms in the figures represent means and the bars standard errors of between 3 and 5 experiments carried out in triplicate.

Since GSH levels often varied between experiments, due to cell culture conditions, the results were therefore normalised to control level before analysis was carried out. In these cases, the control levels therefore have no standard error.

Chapter 3 Epithelial characteristics of A549 and 16HBE cells and the role of GSH in cell proliferation.

3.1 INTRODUCTION

The two cell types used throughout this thesis were the A549 and the 16HBE cell lines. A549 cells are a tumour cell line (Lieber et al., 1976), initiated from a human alveolar cell carcinoma, and were originally shown to have properties typical of human alveolar type II cells. These include the presence of multilamellar cytoplasmic inclusion bodies and the ability to produce surfactant. It has recently been shown, however that they no longer retain the ability to produce surfactant (Li et al., 1995). A549 cells are thus only used as a model of 'alveolar-like' cells which, when grown in culture, still retain the characteristic cobble shape. 16HBE cells are SV40 transformed primary bronchial cultured epithelial cells and have been shown to retain differential epithelial morphology. Monolayers of these cells have been shown (Cozens et al., 1994) to generate transepithelial resistance, have tight junctions and cells grown in a liquid/air interface retain the ability to generate cilia, properties typical of human bronchial cells. 16HBE cells when grown in culture also retain the characteristic cobble shape, but unlike the A549 cells, which tend to grow individually, they grow in clumps, requiring cell-cell contact for growth.

3.2 CHARACTERISATION OF THE EPITHELIAL CELL LINES:

In order to show that both cells retained epithelial properties, they were stained with a mouse anti-human cytokeratin antibody that is known to react with human epithelial cells. The cells were then visualised by the immunoperoxidase method as described in 2.2.5 and the nuclei were stained using nuclear fast red. Figures 2 and 3 show that both cell types stained positive for epithelial characteristics.

Figure 2



Figure 3



Figures 2 and 3: A549 (figure 2) and 16HBE (figure 3) cells stained with the cytokeratin antibody. (C) Corresponds to the cytokeratin and (N) to the nucleus.

3.3 CELL CONFLUENCY.

Throughout this thesis the term confluency is used to describe a point in cell growth when the well surface becomes completely covered by cells. In all experiments described herein cells were treated at 70% confluency, which refers to the point at which cells covered approximately 70% of the well surface. This measurement, although subjective, ensured that cells were treated at approximately the same state for each experiment. Figures 4 and 5 give an indication of the degree of confluency for both cell types. Cells were grown in monolayers to each confluency level, trypsinised and counted using a haemocytometer. This was carried out several times to ensure that the level of confluency produced consistent cell numbers between different experiments. Cell numbers were found to be similar for both cell types on each occasion (Table 1).

Table 1 The Number of Cells at Different Confluency Points in both A549 and 16HBE cells.

% confluency	Number of A549 cells	Number of 16HBE cells
70%	1.4×10^6	1.5×10^6
85%	1.7×10^6	1.6×10^6
95%	2.1×10^6	2.12×10^6
99%	2.4×10^6	2.3×10^6

For experimental purposes, cells were normally harvested before they reached 100% confluency, i.e. they were harvested between 95 and 99% confluencies. This was because once cells had formed a confluent monolayer they began to grow on top of one another and floated off into the medium. Furthermore, intracellular GSH levels decreased after cells had reached confluency.

Figure 4

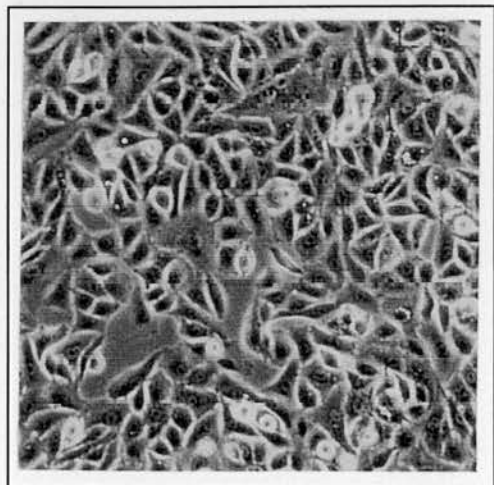
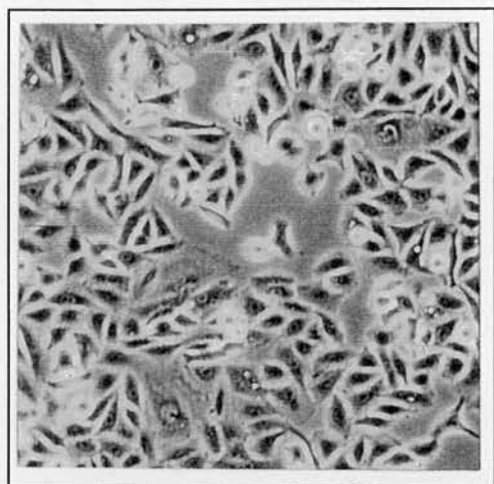
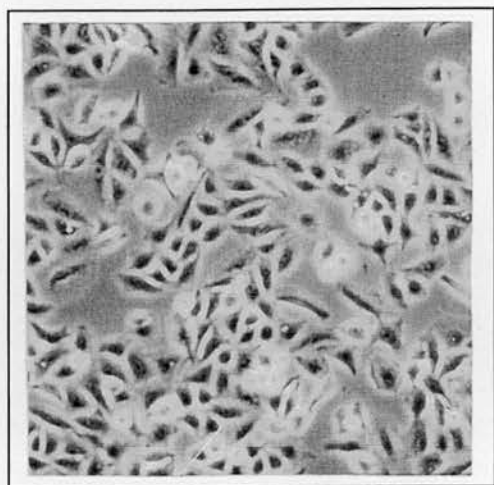
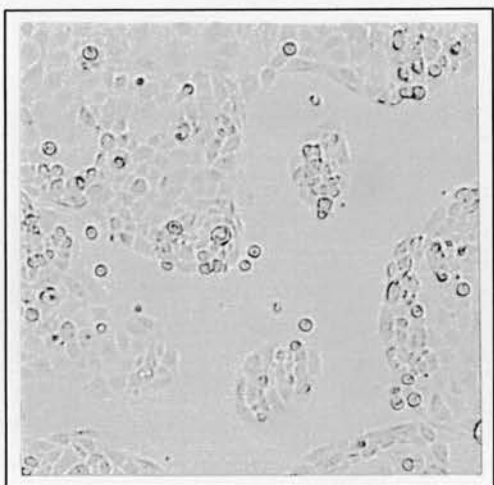
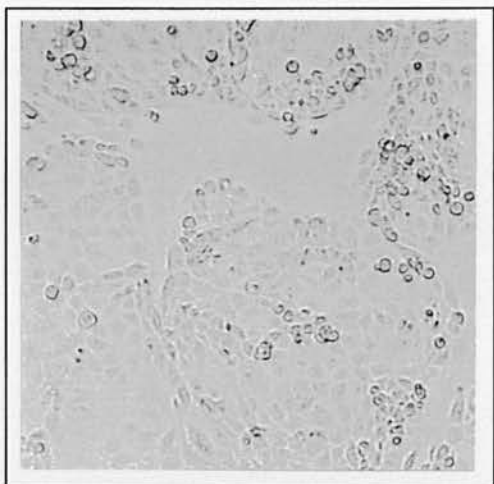


Figure 5

a



b



c

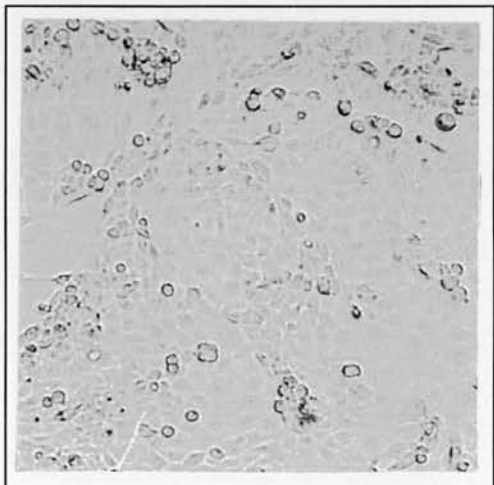


Figure 4 and 5: the appearance of A549 (figure 4) and 16HBE (figure 5) cells at **a**: 70% confluency, **b**: 85% confluency and **c**: 99% confluency.

3.4 REDUCED GSH LEVELS IN THE EPITHELIAL CELL LINES.

In preliminary studies total intracellular GSH levels varied considerably in these cell lines from experiment to experiment. A number of experiments were therefore carried out in both epithelial cell lines to determine if there was a period of time in the growth of the cells when intracellular GSH levels were stable. Cells were seeded at fairly low densities and harvested at different percentage confluencies, counted and total intracellular GSH levels measured. Cells were also grown in varying culture conditions in order to determine if serum concentrations had any effect on GSH levels. In these experiments, cells were grown in complete medium, harvested and subcultured into 6 well plates, again in complete medium. When the cells had reached 30% confluency the medium was removed and replaced with complete medium (10% FBS), or medium supplemented with 2% BSA. Cells were then harvested at different percentages of confluency and the intracellular GSH levels measured. Figures 6 and 7 show the effects of different culture conditions and different confluencies on GSH levels. 110% represents cells grown to confluency and left for a further 24 hours. GSH levels increased in both cell types to 70% confluency, at which point they remained relatively stable until they had formed a confluent monolayer (99%), and thereafter dropped significantly (110%). No significant difference was observed between cells grown in 10% FBS and 2% BSA.

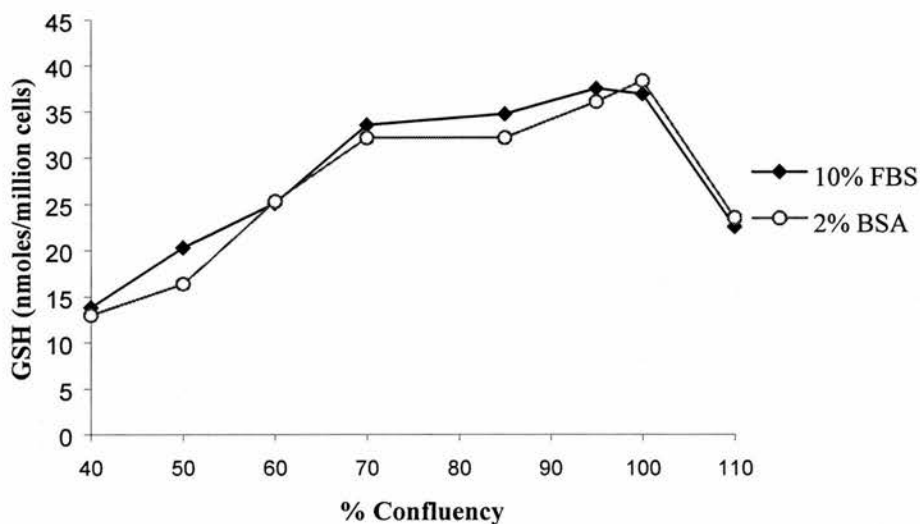


Figure 6: The effect of different culture conditions and confluency on intracellular GSH levels in A549 cells. The symbols represent the means, SEMs are absent for clarity. GSH levels below 70% confluency were significantly different to those at 70% confluency ($p<0.05$), 85% ($p<0.05$), 95% ($p<0.01$) and 100% ($p<0.01$). Cells grown to 110% confluency were significantly different ($p<0.05$) compared to 100% confluency. There was no significant difference in GSH levels between 70% and 100% confluency.

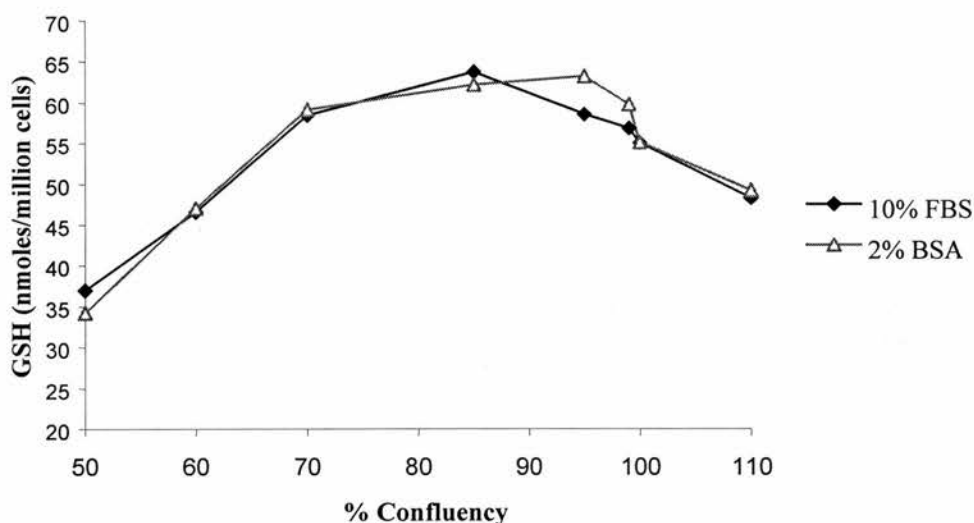


Figure 7: The effect of different culture conditions and confluency on intracellular GSH levels in 16HBE cells. The symbols represent the means, SEMs are absent for clarity. GSH levels below 70% confluency were significantly different to those at 70% confluency ($p < 0.01$), 85% ($p < 0.01$), 95% ($p < 0.01$) and 100% ($p < 0.01$). Cells grown to 110% confluency were not significantly different compared to 100% confluency. There was no significant difference in GSH levels between 70% and 100% confluency but there is a significant difference ($p < 0.05$) between 95% and 110% confluency.

It is a normal experimental procedure to quiesce cells for 24 hours before using them for experimental manipulation. I therefore measured GSH levels in cells following replacement of the serum-supplemented medium with serum free medium. Figures 8 and 9 show the effects of growing cells to 70% confluency in 2% serum (BSA) supplemented medium, followed by removal of medium over a 24 hours period. GSH levels decreased significantly in both cell types following removal of serum, although this effect was more significant in A549 cells. Removal of serum also resulted in a decrease in cell proliferation; cells containing serum reached 95% confluency after 24 hours whereas cells without serum only reached approximately 80% confluency over 24 hours.

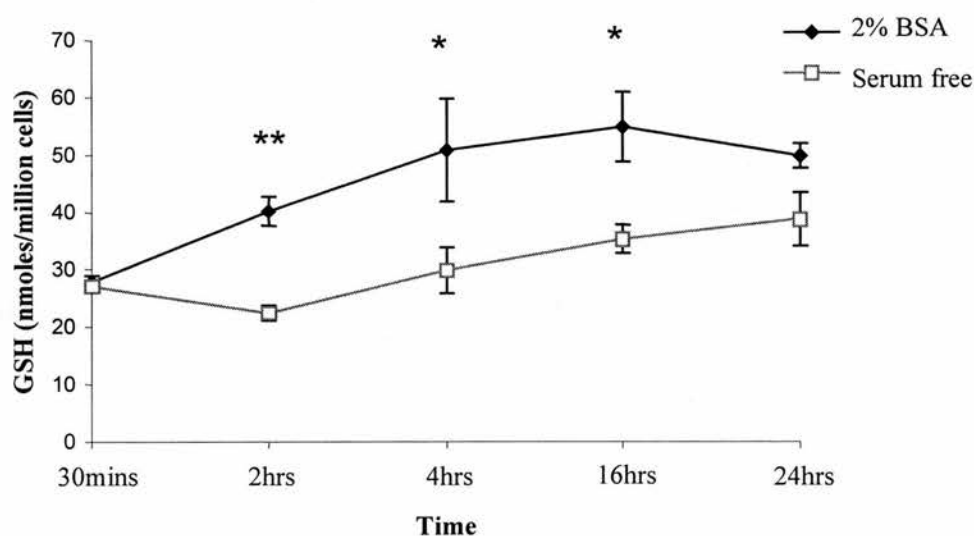


Figure 8: The effect of 2% BSA supplemented medium and serum free medium on intracellular GSH in A549 cells at different time points after serum removal. The points represent means and bars SEMs. (** $p < 0.01$, * $p < 0.05$ compared to serum free at the same time.)

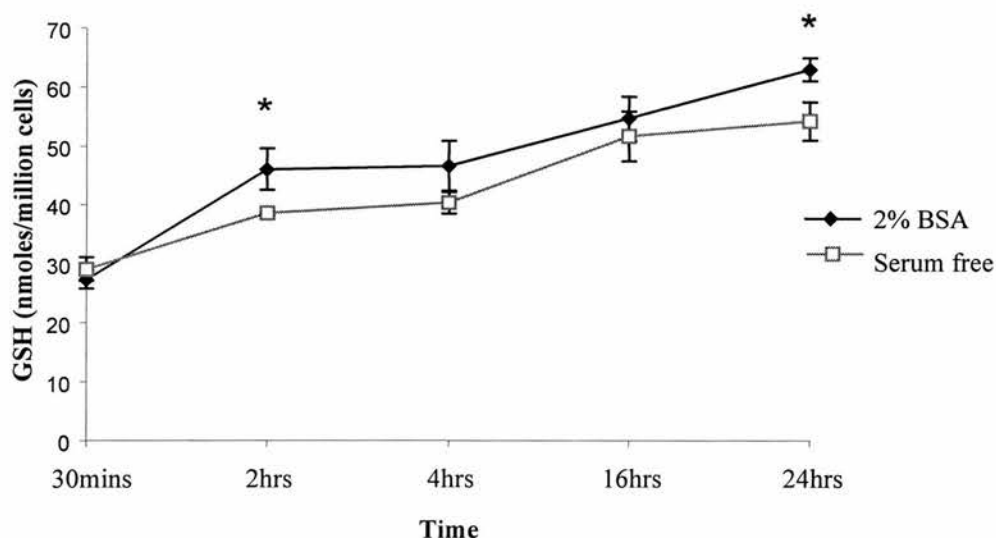


Figure 9: The effect of 2% BSA supplemented medium and serum free medium on intracellular GSH in 16HBE cells at different time points after serum removal. The points represent means and bars SEMs. (* $p < 0.05$ compared to serum free at the same time.)

It has also been observed that at different passage numbers, cells had different intracellular non-protein thiol (NPSH) levels. GSH constitutes 90% of the intracellular non-protein thiol (NPSH) levels, and therefore NPSH levels reflect intracellular GSH levels. Figures 10 and 11 show the NPSH levels of both A549 and 16HBE cells for different passage numbers. Based on these results, which were obtained by Dr. B. Mulier, A549 cells were always used between passage numbers 41 to 80 and 16HBE cells were used between passage numbers 10 to 25, to ensure stable GSH levels.

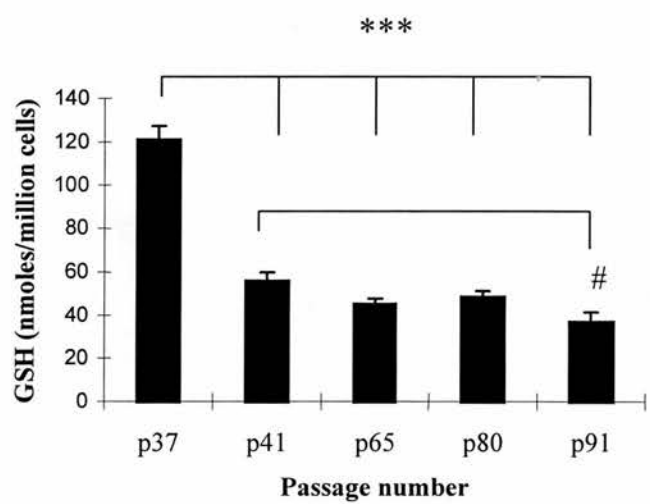


Fig 10: The levels of intracellular NPSH for different passage numbers in A549 cells. Histograms represent means and bars SEMs. (***) $p < 0.001$ compared to p37, # $p < 0.05$ compared to p41).

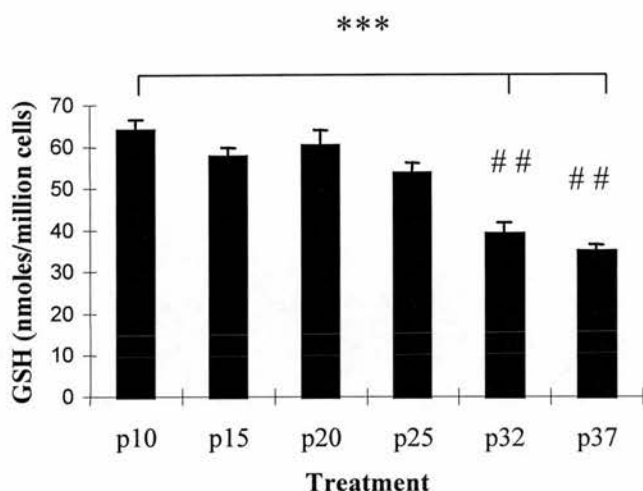


Fig 11: The levels of intracellular NPSH for different passage numbers in 16HBE cells. Histograms represent means and bars SEMs. (***) $p < 0.001$ compared to p10, ## $p < 0.05$ compared to all other passages)

3.5 SUMMARY.

- Both A549 and 16HBE cells retain epithelial properties, as shown by cytokeratin positivity.
- GSH levels are at their most stable between 70 and 99% confluency in both A549 and 16HBE cells.
- The removal of serum from cell medium results in intracellular GSH levels decreasing over time and this decrease is also associated with a decrease in cell proliferation.
- To obtain stable intracellular NPSH levels, and hence GSH levels, A549 cells should be used between passage 41 to 80, and 16HBE cells should be used between passage 10 to 25.

3.6 DISCUSSION.

Previous studies have shown that intracellular GSH levels are affected by culture conditions (Kang and Enger, 1990; Post et al., 1983). Post *et al* (Post et al., 1983)

showed that GSH levels of A549 cells vary after they are passaged, levels increasing up to 24 hours and decreasing thereafter. Post and colleagues also showed that serum levels in the medium affect intracellular GSH levels, increased serum concentrations increased GSH levels, and the absence of serum correlated with decreased GSH levels. My results show that cells grown in serum supplemented medium have fairly stable intracellular GSH levels between 70% and 99% confluency, which can occur up to 48 hours after passaging, and no significant difference was observed between cells grown in 10% serum and 2% serum. However, the data described here and that of Post *et al* cannot be directly compared since cell densities were not recorded by Post *et al*. My data show that once cells had formed a confluent monolayer however, GSH levels decreased, possibly due to cells entering the G₁ or G₀ plateau phase, i.e. the cells cease to proliferate. Cells grown in medium lacking serum did, however, have lower GSH levels compared to cells grown in serum supplemented medium. These cells also had a much slower growth rate, taking up to 2 days longer to reach confluency, possibly, again, due to cells being in a G₁ or G₀ plateau growth phase. Cells grown in serum free medium are known as quiesced cells, and it is thought that cells contain several molecules which are critical in allowing cells to pass through the restriction stage in the cell cycle, the G₁ stage. Serum deprivation is thought to destroy these molecules and hence the cells remain in the G₁ stage, which, in quiesced cells is referred to as the G₀ stage (Alberts et al., 1989). It has also been shown (Harris and Teng, 1997) that GSH levels are fairly constant through the cell cycle except when cells are grown to a G₁ or G₀ plateau phase in either exhausted or serum deficient medium. Furthermore Kang and Enger (Kang and Enger, 1990) observed that A549 cells contained a higher amount of GSH in the early portion of the exponential phase of growth, and lower levels at the plateau phase of growth. These studies (Harris and Teng, 1997; Kang and Enger, 1990; Post et al., 1983) suggest that GSH is involved in the regulation of cell growth. In support of this, treatment of A549 cells with butathione sulphoximine (BSO), which depletes GSH levels, is also associated with a decrease in cell growth (Kang and Enger, 1990) and this decrease is dose dependent. BSO is not toxic to the cells since removal of BSO results in cell proliferation (Kang and Enger, 1992). This is consistent with results described herein since intracellular GSH levels begin to fall after 100% confluency, a

point at which cell growth is much slower, suggesting that GSH levels and proliferation are related. However, it has been shown that the decrease in GSH does not correlate with proliferation, (Kang, 1993) and it is thought that BSO actually inhibits proliferation by inhibiting cysteine uptake, which is necessary for cell growth and de novo GSH synthesis. Addition of extracellular GSH can partially overcome the effects of BSO on GSH levels, and for extracellular GSH to have this effect it needs to be broken down into its constitutive amino acids, one of which is cysteine. Poot et al (Poot et al., 1995) have shown that the decrease in proliferation following BSO treatment results in cells arresting in the S and G₂ phase of the cell cycle, which is consistent with the hypothesis that GSH synthesis is required for cell growth. Cells sub-cultured during exponential growth also contain higher levels of GSH. The critical role of thiols, especially GSH, in cell proliferation, has also been observed in human bronchial epithelial cells, (Atzori et al., 1994) where GSH is thought to play a part in transmembrane signal transduction by modifying the redox status of critical proteins which are involved in proliferation. Thus, it is possible that GSH is a candidate for one of the essential molecules that are required for cell cycling (Alberts et al., 1989), since removal of serum causes both a decrease in intracellular GSH levels, and an arrest of cells in the G₁ plateau phase known as G₀.

My results, taken together with other studies, suggest that lung epithelial cells should be grown in serum supplemented medium and used at a stage in which they are still proliferating. This should ensure that GSH levels are relatively stable during experimental interventions. Since I wish to observe changes in GSH in both cell types following various treatments, I treated cells at 70% confluency, harvested them at between 90-99% confluency and always treated them in serum supplemented medium.

Chapter 4 Induction of Nitric Oxide in Both A549 and 16HBE Cells and the Effect of Nitric Oxide Induction on GSH Levels.

4.1 INTRODUCTION.

NO[•] can be induced in many different cell types, including epithelial cells (Robbins et al., 1994;), macrophages (Steuhr and Marletta, 1987), hepatocytes (Geller et al., 1993) and endothelial cells (Murphay et al., 1991) using a variety of stimuli such as TNF- α , IL1- β , IFN- γ , and LPS. Inducible nitric oxide synthase (iNOS), unlike the constitutive cNOS is switched on by these stimuli, resulting in an increase in NO[•] release from cells. This increase can be measured in cell medium using the Greiss assay which measures nitrite levels. NO[•] reacts with molecular oxygen, abstracting a single electron, resulting in the formation of nitrite (NO₂⁻), a product which is easily measured in the cell medium. However, the assay appears to be limited and only indicates that iNOS has been switched on, resulting in an increase in nitrite in the medium. In the results that follow, the assay does not appear to show differences above a certain level (approximately 0.6 μ M/million cells). This may be due to nitrite itself being oxidised to give nitrate and a further assay can be carried out in order to determine levels of both nitrite and nitrate (Green et al., 1982). This was not carried out in our experiments, however, since we carried out further assays (PCR) in order to assess iNOS induction. The oxidation of nitrite to nitrate may therefore be a limitation in the accuracy of the Greiss assay in measuring NO[•] release.

4.2 INDUCTION OF NO[•] AND NITRITE PRODUCTION IN EPITHELIAL CELLS IN RESPONSE TO CYTOKINES AND LPS.

NO[•] induction, as measured by both nitrite levels in the medium and iNOS mRNA has previously been shown for A549 cells (Robbins et al., 1994) using cytomix (TNF- α , IL1- β , and IFN- γ , 10ng/ml of each). I therefore determined the

concentration of cytomix required in our system to induce nitrite production in both A549 and 16HBE cells. Figures 12 and 13 show the effects of cytomix at concentrations of both 10ng/ml and 20ng/ml of each cytokine. Cytomix was diluted in 2% medium and added to the cells once they had reached 70% confluency. Nitrite levels were measured 24 hours later. Ten ng/ml appears to be sufficient for the induction of nitrite production (causing a 300% increase) and there is no added effect of using the higher concentration of 20ng/ml in either cell type. This concentration of cytomix (10ng/ml) was thus used throughout the thesis unless otherwise stated.

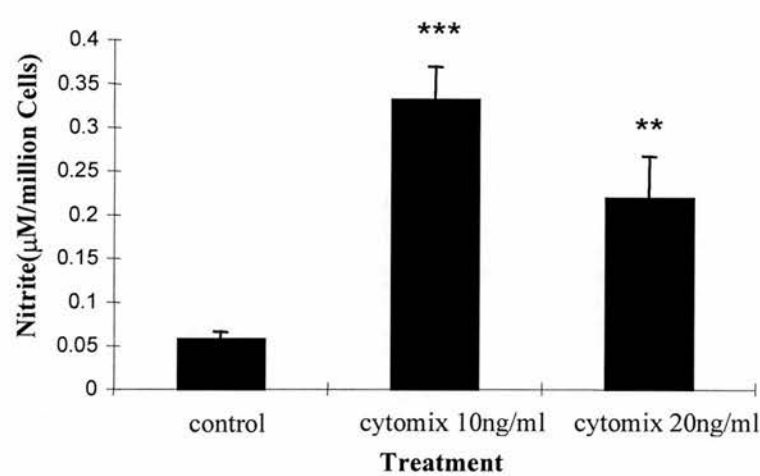


Figure 12: The effect of different concentrations of cytomix on nitrite production by A549 cells at 24hrs. Histograms represent means and bars SEMs. (**p < 0.01, ***p < 0.001, compared with control).

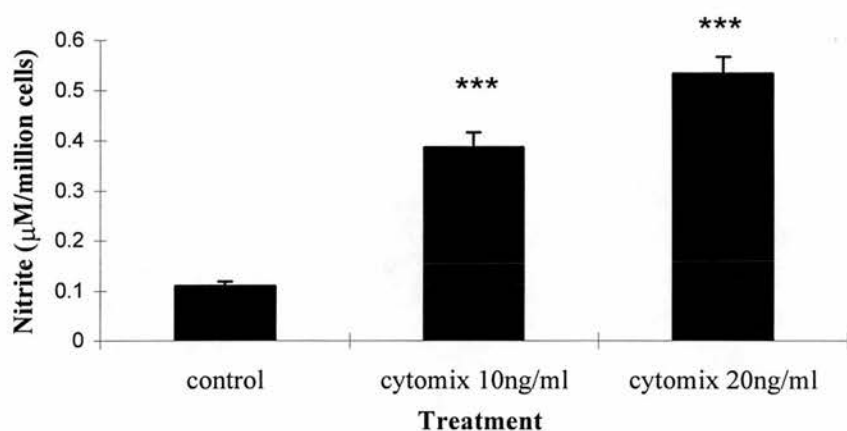


Figure 13: The effect of different concentrations of cytomix on nitrite production by 16HBE cells at 24hrs. Histograms represent means and bars SEMs. (***) $p < 0.001$, compared with control).

The time course of nitrite production was carried out to determine the optimal time for measuring nitrite following treatment with cytomix. Cells were treated with cytomix and nitrite levels were measured at 8 hourly intervals. The earliest time point at which nitrite production was significantly different from control was 24 hours. Figures 14 and 15 show the time course of nitrite production over 24hrs in both A549 and 16HBE cells.

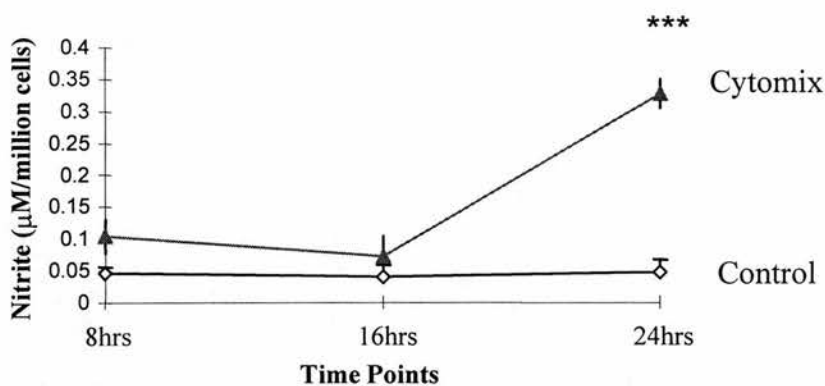


Figure 14: The effect of cytomix on nitrite production in A549 cells over a period of 24hrs. Symbols represent means and bars SEMs. (***) $p < 0.001$ compared with control).

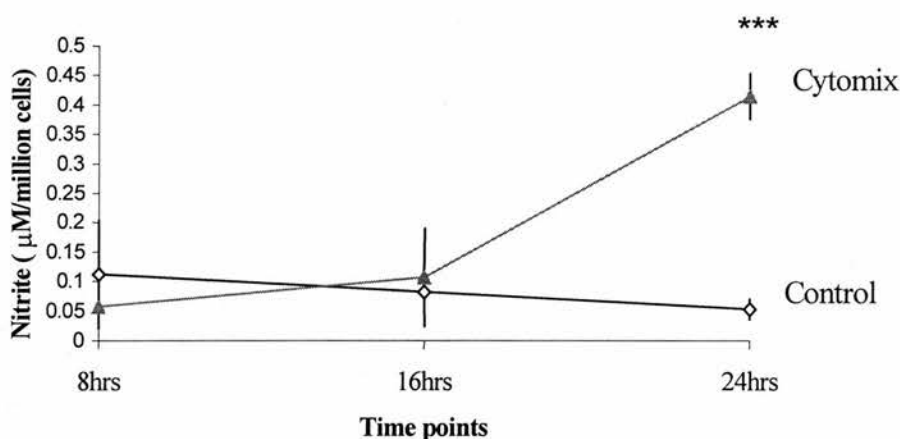


Figure 15: The effect of cytomix on nitrite production in 16HBE cells over a period of 24hrs. Symbols represent means and bars SEMs. (***) $p < 0.001$ compared with control).

Since cytomix has been used previously to induce NO^- in A549 cell, (Adcock et al., 1994; Robbins et al., 1994). I examined the effects of the individual cytokines on nitrite production. Figures 16 and 17 show the effect of the single cytokines ($\text{TNF-}\alpha$, $\text{IL1-}\beta$, $\text{IFN-}\gamma$, all at 10ng/ml) on nitrite production in both cell types. $\text{TNF-}\alpha$ causes a significant increase in nitrite production in both A549 and 16HBE cells, although

nitrite levels were significantly lower than those observed with cytomix. There was no significant increase in nitrite levels following addition of the other cytokines (IL1- β and IFN- γ) and there was a significantly higher level of nitrite following cytomix than each individual cytokine. The combination of cytomix was therefore used throughout this thesis since it was a significantly stronger signal than TNF- α alone.

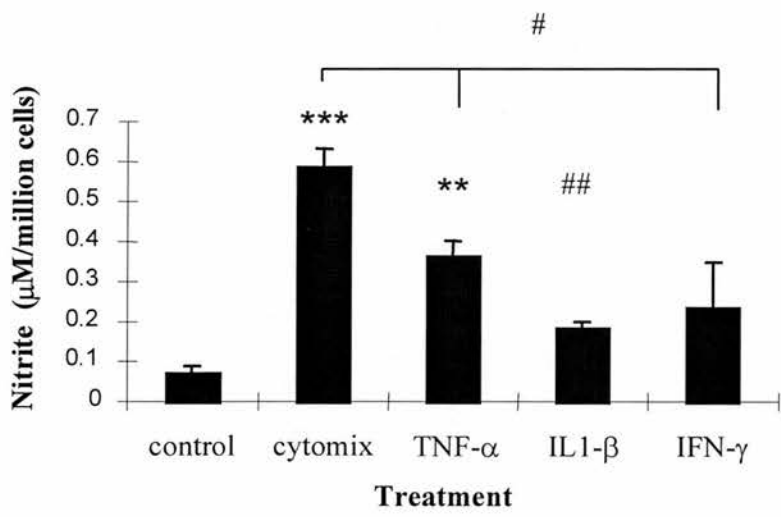


Figure 16: The effect of individual cytokines on nitrite production in A549 cells. Histograms represent means and bars SEMs.(***p<0.001, **p<0.01, compared to control, #p<0.05, ##p<0.01, compared to cytomix).

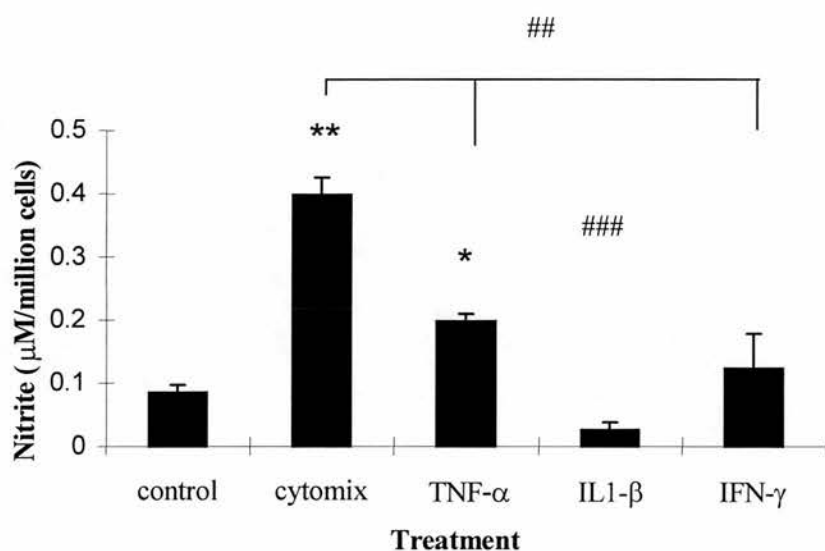


Figure 17: The effect of individual cytokines on nitrite production in 16HBE cells. Histograms represent means and bars SEMs. (**p<0.01, *p<0.05, compared to control, ##p<0.01, ###p<0.001 compared to cytomix).

LPS has also been used in a variety of cell types to induce iNOS and NO release (Chesrown et al., 1994; Steuhr and Marletta, 1987). I therefore examined the effect of LPS alone and in combination with cytomix. Figures 18 and 19 show the effect of LPS (1μg/ml) on nitrite production in both cell types. There was no significant increase in nitrite levels following addition of LPS to A549 cells, but there was a significant increase in nitrite levels in 16HBE cells following addition of LPS. There was no additive effect of cytomix and LPS on nitrite release in either cell type.

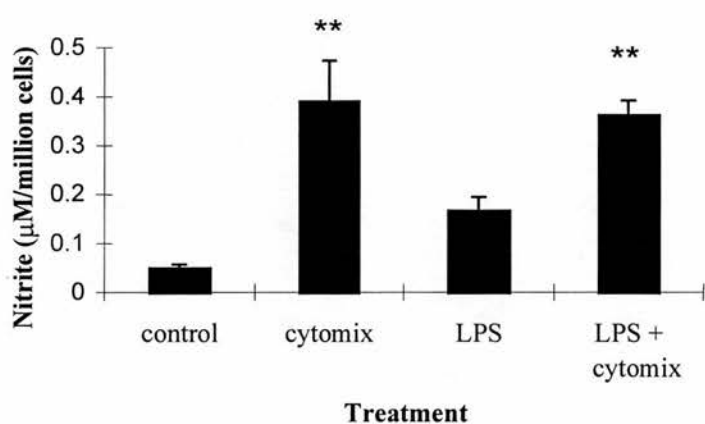


Figure 18: The effect of LPS on nitrite production in A549 cells. Histograms represent means and bars SEMs. (**p<0.01 compared to control).

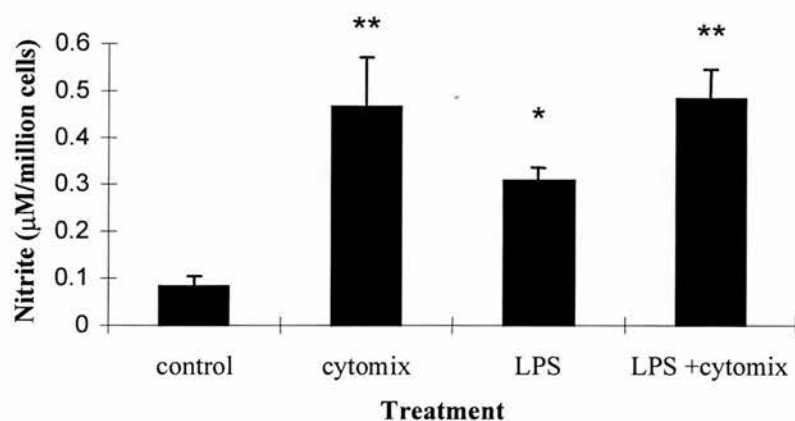


Figure 19: The effect of LPS on nitrite production in 16HBE cells. Histograms represent means and bars SEMs. (**p<0.01, *p<0.05, compared to control).

4.3 INHIBITION OF NO[•] RELEASE BY THE NOS INHIBITOR LN^G-MONOMETHYL-L-ARGININE (L-NMMA).

In order to show that the data presented above are as a result of nitrite production, and hence induction of NOS, the inhibitor L-NMMA was used. L-NMMA is an analogue of L-arginine and prevents NO[•] formation by competitive inhibition of the enzyme NOS. Cells were pre-treated with L-NMMA for 1 hour followed by treatment with cytomix for a further 24 hours. Figures 20 and 21 show that pre-treatment with L-NMMA significantly decreased nitrite production in both cell types following cytokine treatment.

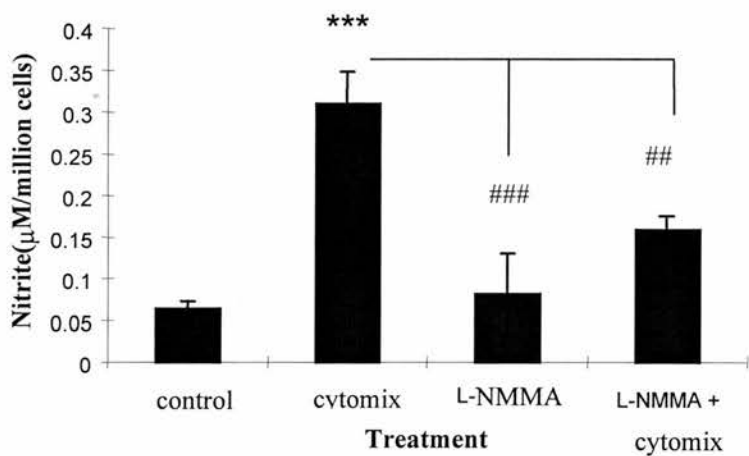


Figure 20: The effect of pre-treating A549 cells for 1hr with L-NMMA followed by treatment with cytomix for 24hrs. Histograms represent means and bars SEMs. (***) $p < 0.001$, compared with control, (##) $p < 0.01$, (###) $p < 0.001$, compared with cytomix.)

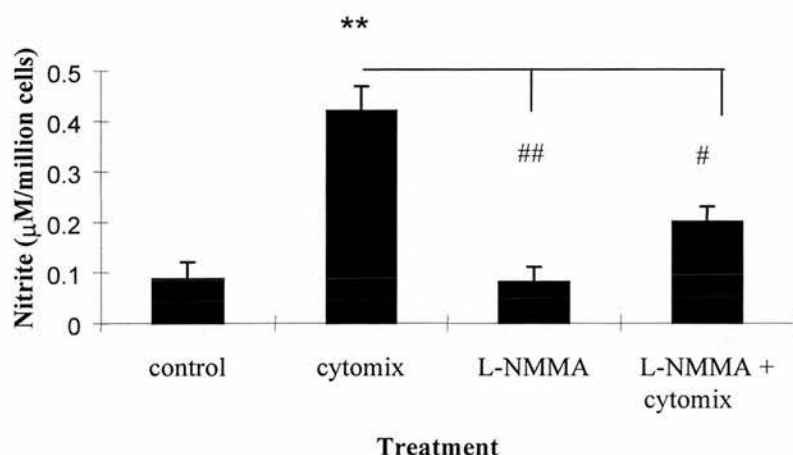


Figure 21: The effect of pre-treating 16HBE cells for 1hr with L-NMMA followed by treatment with cytomix for 24hrs. Histograms represent means and bars SEMs. (** $p < 0.01$, compared with control, # $p < 0.05$, ## $p < 0.01$, compared with cytomix.)

4.4 INDUCTION OF NO[•] USING THE OXIDANT H₂O₂.

In the airway an initial response to oxidant stress is thought to be the induction of NF- κ B responsive genes such as iNOS. I therefore examined the effect of H₂O₂ on NO[•] release. H₂O₂ is an oxidant and can cause cell damage in high concentrations (Mulier et al., 1998). Three concentrations were chosen, of which the highest (10 μ M) has previously been shown to be non-toxic in A549 cells. Toxicity was previously measured as the amount of LDH release (Mulier et al., 1998), which gives an indication of cell membrane destruction and cell death. Trypan blue exclusion was also carried out in the three treatments. Figures 22 and 23 show a dose response ranging from 100nM to 10 μ M, of H₂O₂, in 2% medium on nitrite production in both A549 and 16HBE cells. Nitrite production increased significantly in both cells following treatment with all three concentrations of H₂O₂. In subsequent experiments I used 1 μ M of H₂O₂ since this concentration, although not significantly different to the other concentrations, tended to produce higher (>400% increase) levels of nitrite.

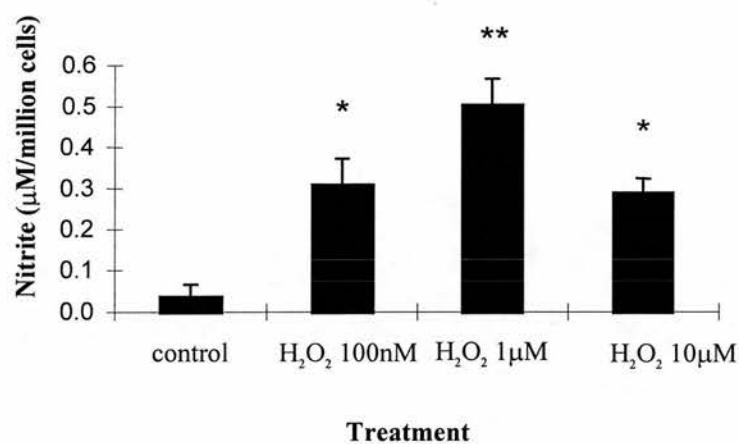


Figure 22: Dose response of H₂O₂ on nitrite production in A549 cells. Histograms represent means and bars SEMs. (*p<0.05,**p<0.01 compared to control)

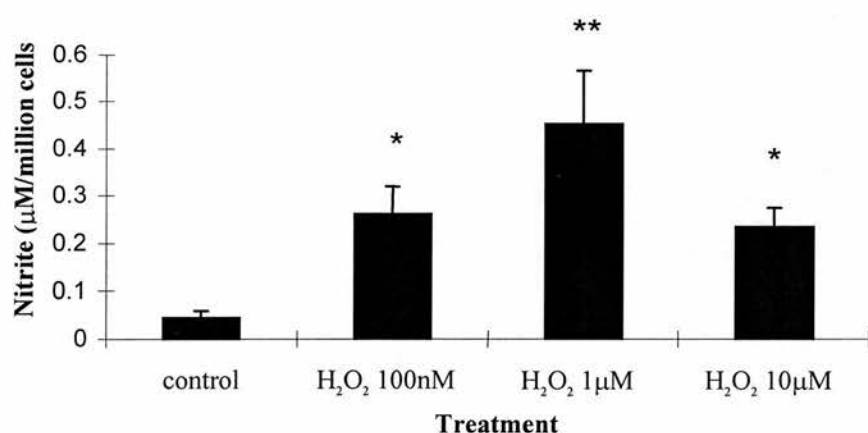


Figure 23: Dose response of H₂O₂ on nitrite production in 16HBE cells. Histograms represent means and bars SEMs. (*p<0.05,**p<0.01 compared to control)

Figures 24 and 25 show the additive effect of both H₂O₂ and cytomix on nitrite release. No significant additive effect was observed in either cell type following this combination of NO[•] inducers. This may either be due to limitations of the assay, or formation of nitrate which has not been measured (see discussion).

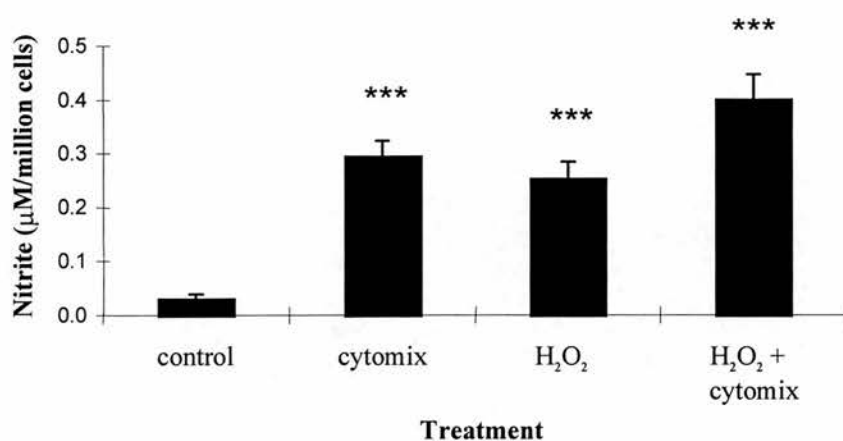


Figure 24: The effect of cytomix, H₂O₂ and a combination of cytomix and H₂O₂ on nitrite production in A549 cells. Histograms represent means and bars SEMs. (***) $p < 0.001$ compared to control)

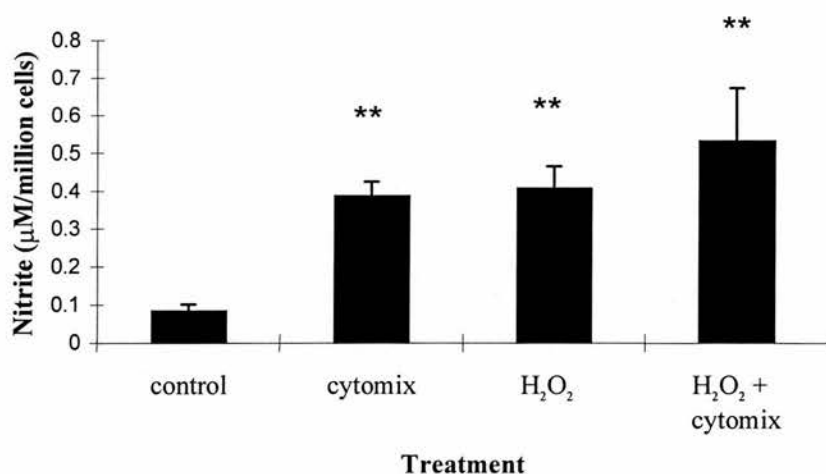


Figure 25: The effect of cytomix, H₂O₂ and a combination of cytomix and H₂O₂ on nitrite production in 16HBE cells. Histograms represent means and bars SEMs. (**) $p < 0.001$ compared to control).

The NOS inhibitor L-NMMA was used to show that the observed effects of H₂O₂ on nitrite production was due to the induction of iNOS and subsequent NO[•] release. Figures 26 and 27 show the effect of pre-treatment with L-NMMA for 1 hour

followed by H_2O_2 treatment for 24 hours in both cell types. In both cell types, pre-treatment with L-NMMA significantly decreased nitrite production following H_2O_2 treatment.

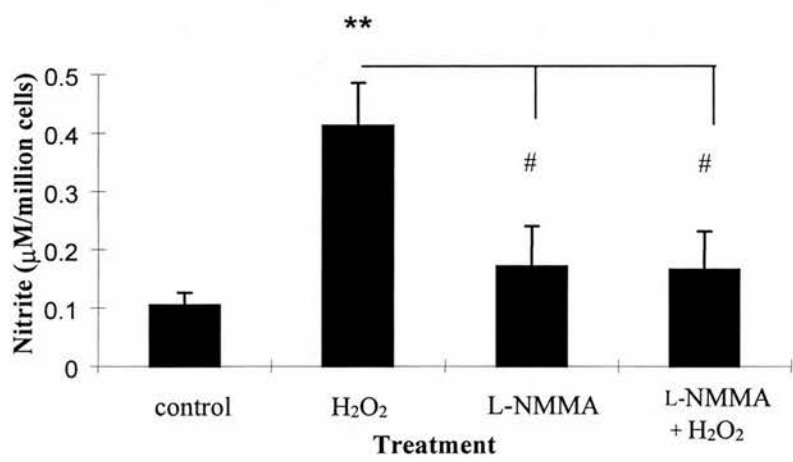


Figure 26: The effect of pre-treating A549 cells for 1hr with L-NMMA followed by treatment with H_2O_2 for 24hrs. Histograms represent means and bars SEMs. (** $p < 0.01$, compared with control, # $p < 0.05$, compared with H_2O_2 .)

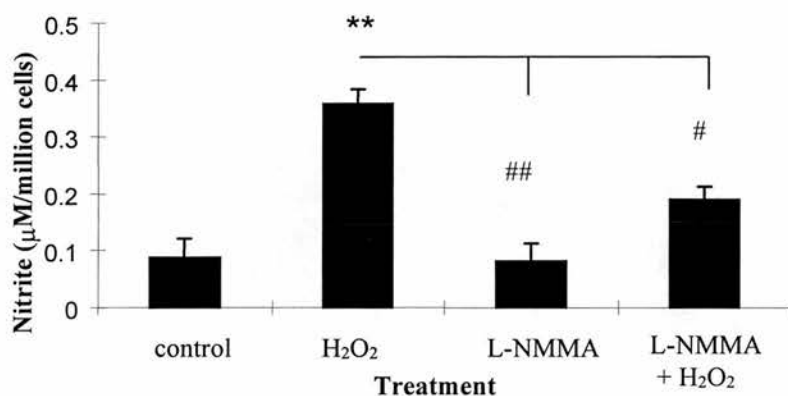


Figure 27: The effect of pre-treating 16HBE cells for 1hr with L-NMMA followed by treatment with H₂O₂ for 24hrs. Histograms represent means and bars SEMs. (**p<0.01, compared with control, #p < 0.05, ##p<0.01 compared with H₂O₂.)

4.5 INTRACELLULAR GSH LEVELS FOLLOWING CYTOMIX AND H₂O₂.

The hypothesis to be tested in this thesis is that the intracellular redox status of the cell, in particular GSH levels, may be important in iNOS induction. GSH levels of both cells were therefore measured following the treatments described above. Figures 28 and 29 show the effect of cytomix at concentrations of 10ng/ml and 20ng/ml on GSH levels in both cell types at 24 hours. In both cell types there is a significant decrease (approximately 20%) in GSH levels following treatment with cytomix. In the 16HBE cells there is also a significant difference between cells treated with the two different concentrations of cytomix, although no difference was observed in nitrite levels between these two concentrations in the 16HBE cells (see figure 13). These results show that a decrease in GSH levels occurs concomitantly with NO[•] release under these conditions. This decrease may be due to either an increase in the oxidant burden of the cell (such as that produced by the action of TNF- α on the mitochondria to release ROS) or due to the formation of nitrosothiols (GSNO) and peroxynitrite.

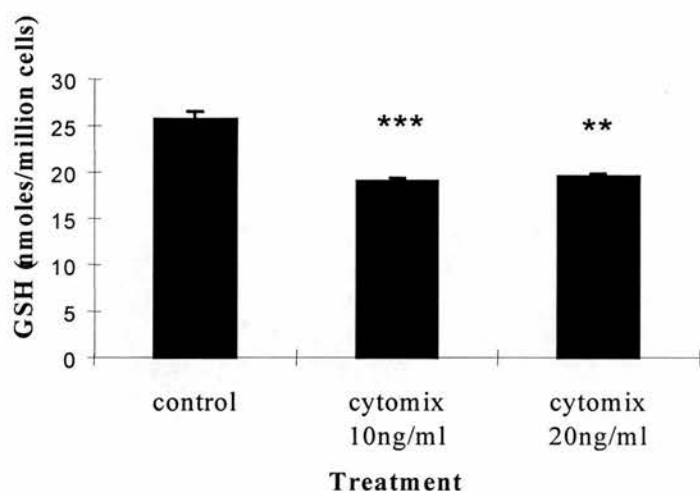


Figure 28: The effect of different concentrations of cytomix on intracellular GSH levels in A549 cells. Histograms represent means and bars SEMs. (**p < 0.01, ***p< 0.001 compared with control)

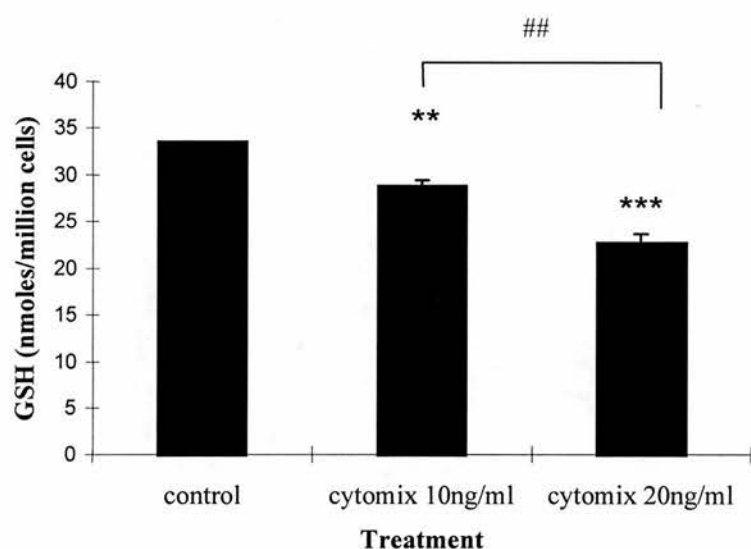


Figure 29: The effect of different concentrations of cytomix on intracellular GSH levels in 16HBE cells. Histograms represent means and bars SEMs. (**p < 0.01, ***p< 0.001 compared with control, ##p<0.01 compared with cytomix 10ng/ml).

Figures 30 and 31 show the effect of the individual cytokines on GSH levels in both cell types. TNF- α causes a significant decrease in GSH levels in both A549 and 16HBE cells, having also produced a significant increase in nitrite levels (see figures

16 and 17). The other two cytokines (IL1- β and IFN- γ) had no significant effect on GSH levels in either cell type.

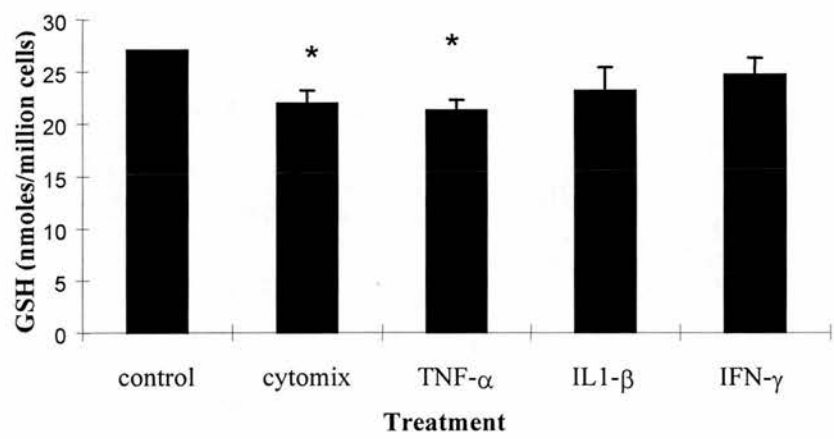


Figure 30: The effect of individual cytokines on intracellular GSH levels in A549 cells. Histograms represent means and bars SEMs. (*p < 0.05, compared to control).

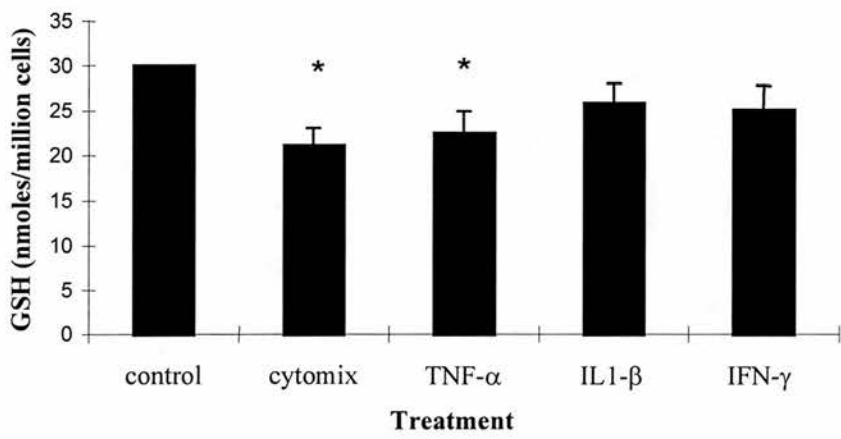


Figure 31: The effect of individual cytokines on intracellular GSH levels in 16HBE cells. Histograms represent means and bars SEMs. (*p < 0.05, compared to control).

Figures 32 and 33 show the effect of LPS on GSH levels in both cell types. LPS caused a significant decrease in GSH levels in 16HBE cells only (having only caused a significant increase in nitrite production in 16HBE cells (see figures 18 and 19)).

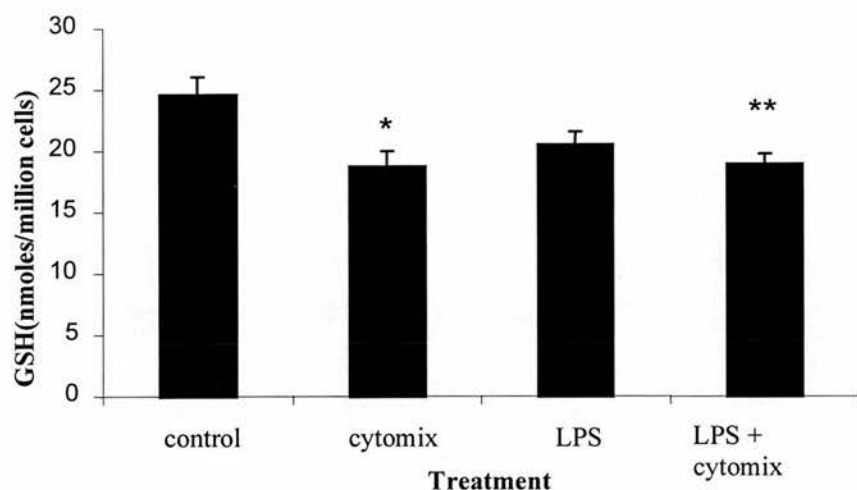


Figure 32: The effect of cytomix and LPS on intracellular GSH levels in A549 cells at 24hrs. Histograms represent means and bars SEMs. (** $p < 0.01$, * $p < 0.05$ compared with control)

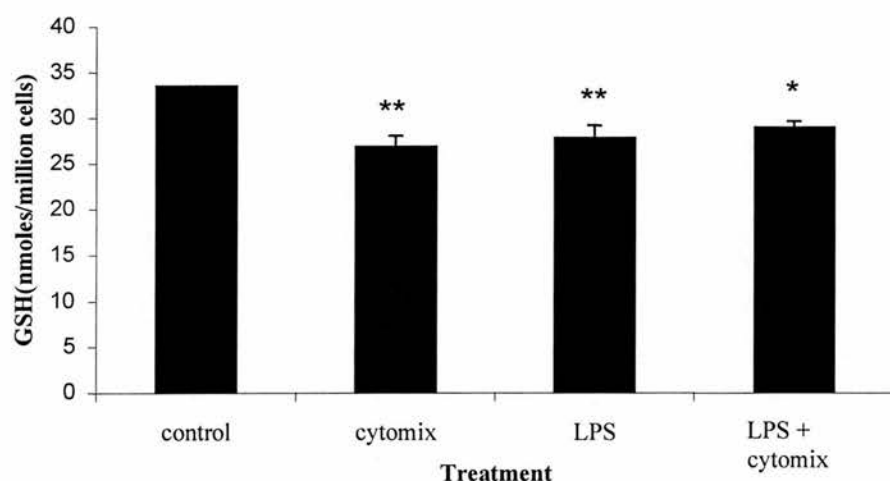


Figure 33: The effect of cytomix and LPS on intracellular GSH levels in 16HBE cells at 24hrs. Histograms represent means and bars SEMs. (** $p < 0.01$, * $p < 0.05$ compared with control)

Figures 34 and 35 show the effect of H_2O_2 on GSH levels in both cell types. A significant decrease in GSH was demonstrated in these cells concomitant with the production of NO . Again there is no additive effect of using cytomix and H_2O_2 .

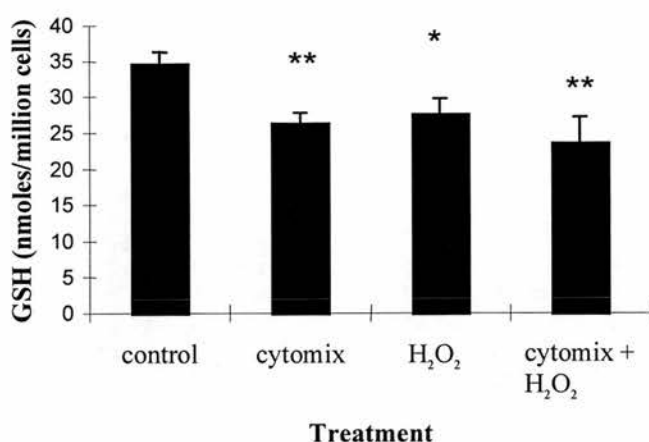


Figure 34: The effect of cytomix and H₂O₂ on intracellular GSH levels in A549 cells at 24hrs. Histograms represent means and bars SEMs. (**p< 0.01, *p< 0.05 compared with control)

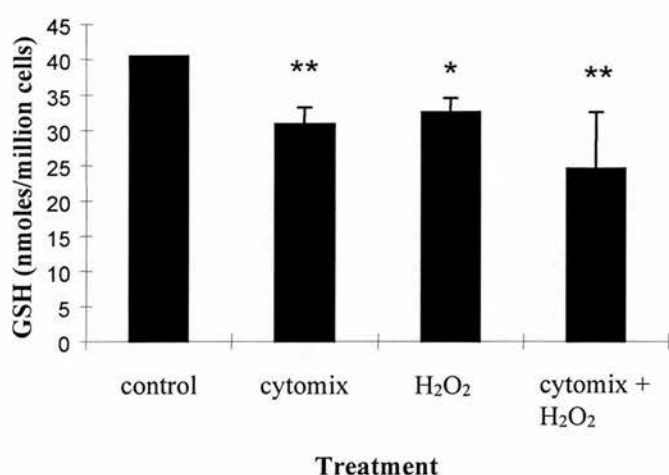


Figure 35: The effect of cytomix and H₂O₂ on intracellular GSH levels in 16HBE cells at 24hrs. Histograms represent means and bars SEMs. (**p< 0.01, *p< 0.05 compared with control).

Figures 36 and 37 show the effect of L-NMMA pre-treatment for 1 hour followed by cytomix and H₂O₂ for 24 hours on GSH levels. In both cell types GSH levels are decreased following pre-treatment with L-NMMA followed by either cytomix or H₂O₂. This would suggest that the decrease in GSH following treatment with

cytomix or H_2O_2 is not due to the formation of GSNO or peroxynitrite but due to an increase in oxidant burden caused by the treatments themselves.

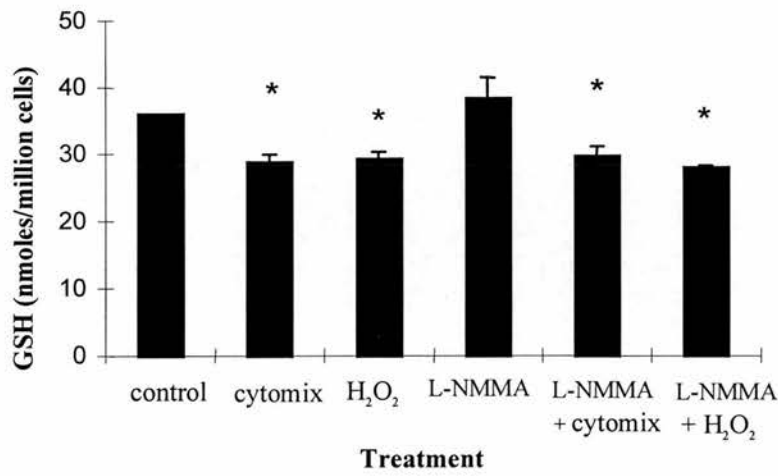


Figure 36: The effect of cytomix, H_2O_2 and L-NMMA on intracellular GSH levels in A549 cells at 24hrs. Cells were pre-treated with L-NMMA for 1 hour followed by treatment with either cytomix or H_2O_2 for 24hrs. Histograms represent means and bars SEMs. (* $p < 0.05$ compared with control)

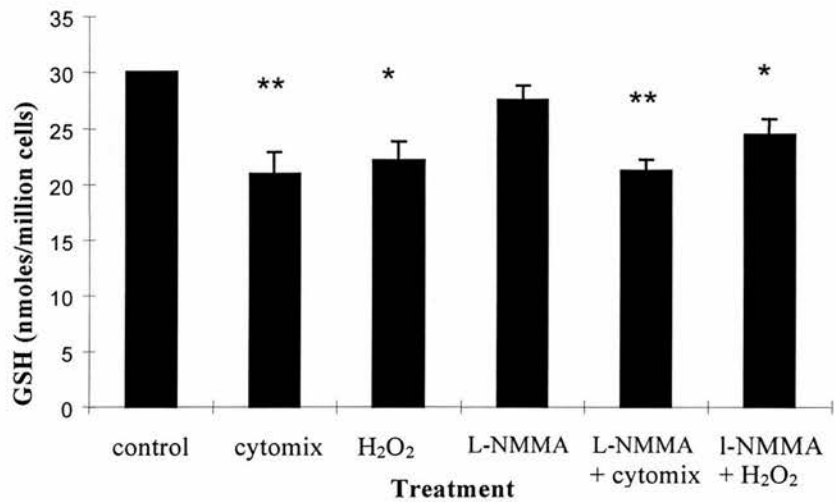


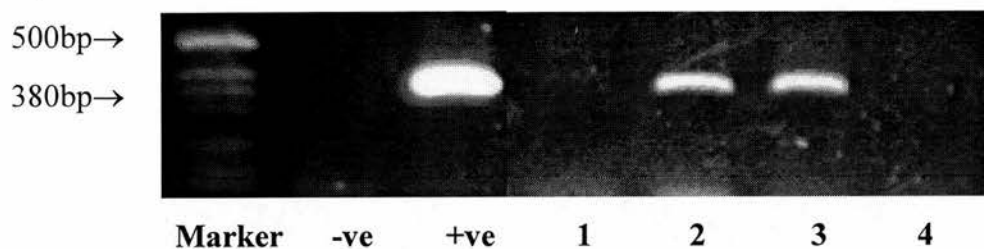
Figure 37: The effect of cytomix, H_2O_2 and L-NMMA on intracellular GSH levels in 16HBE cells at 24hrs. Cells were pre-treated with L-NMMA for 1 hour followed by treatment with either cytomix or H_2O_2 for 24hrs. Histograms represent means and bars SEMs. (* $p < 0.05$, ** $p < 0.01$ compared with control)

4.6 PCR ANALYSIS OF iNOS

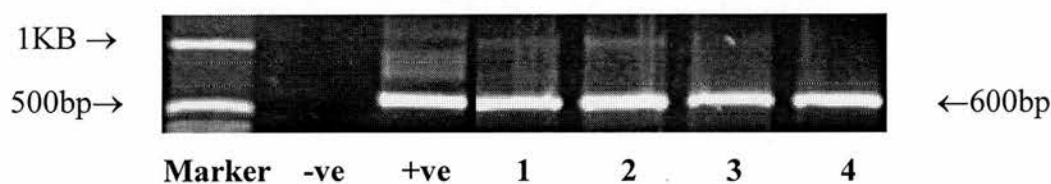
In order to verify that the formation of nitrite was due to the upregulation of iNOS in these cells, iNOS mRNA levels were assessed using the technique of RT-PCR. The housekeeping gene GAPDH was also assessed to allow semi-quantitative analysis of iNOS mRNA expression in the cells. iNOS can be induced at 1- 16 hours (data not shown) and the ratio of iNOS to GAPDH determined. This ratio does not, however, represent the levels of NO[•] produced following the same treatment since it is representative of only one time-point. RT-PCR is therefore only used to indicate whether or not iNOS induction has occurred. Both iNOS and GAPDH levels were quantified using densitometry and levels of iNOS were determined by comparison to levels of GAPDH. A 1 KB DNA ladder was used to determine the band sizes which were 380 bp for iNOS and 600 bp for GAPDH. The first figure shows a positive control for GAPDH to ensure the correct size band is being observed. However since GAPDH is a housekeeping gene its only function in this assay is to determine that the experiment has worked, and hence a positive control was not required for subsequent experiments.

Figure 38 shows the effect of cytomix, H₂O₂ and LPS on iNOS and GAPDH mRNA levels measured by RT-PCR in a) A549 cells and b) 16HBE cells at 4 hours. Both cytomix and H₂O₂ induce iNOS mRNA in both cell types but LPS only induces iNOS in HBE cells.

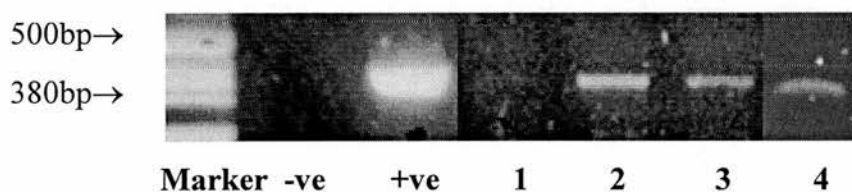
a) iNOS



GAPDH



b) iNOS



GAPDH

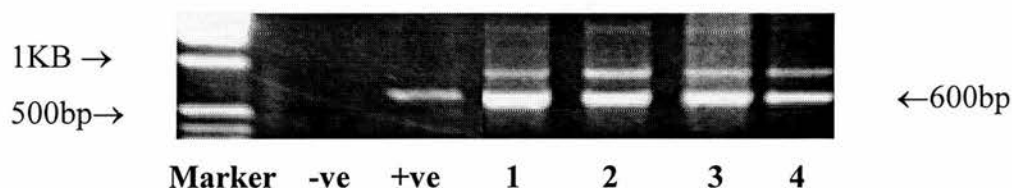


Figure 38: The effect of cytomix, H_2O_2 and LPS on iNOS and GAPDH mRNA levels at 4 hours in a) A549 cells and b) 16HBE cells. -ve control, +ve control, **Lane 1**: control cells, **Lane 2**: cytomix, **Lane 3**: H_2O_2 , **Lane 4**: LPS.

Figure 39 shows the effect of treating both a) A549 and b) 16HBE cells with cytomix, $IL1-\beta$, $TNF-\alpha$ and $IFN-\gamma$ for 4 hours on iNOS and GAPDH mRNA levels. In both cell types both cytomix and $TNF-\alpha$ increase iNOS mRNA levels, but $IL1-\beta$ and $IFN-\gamma$ have no effect.

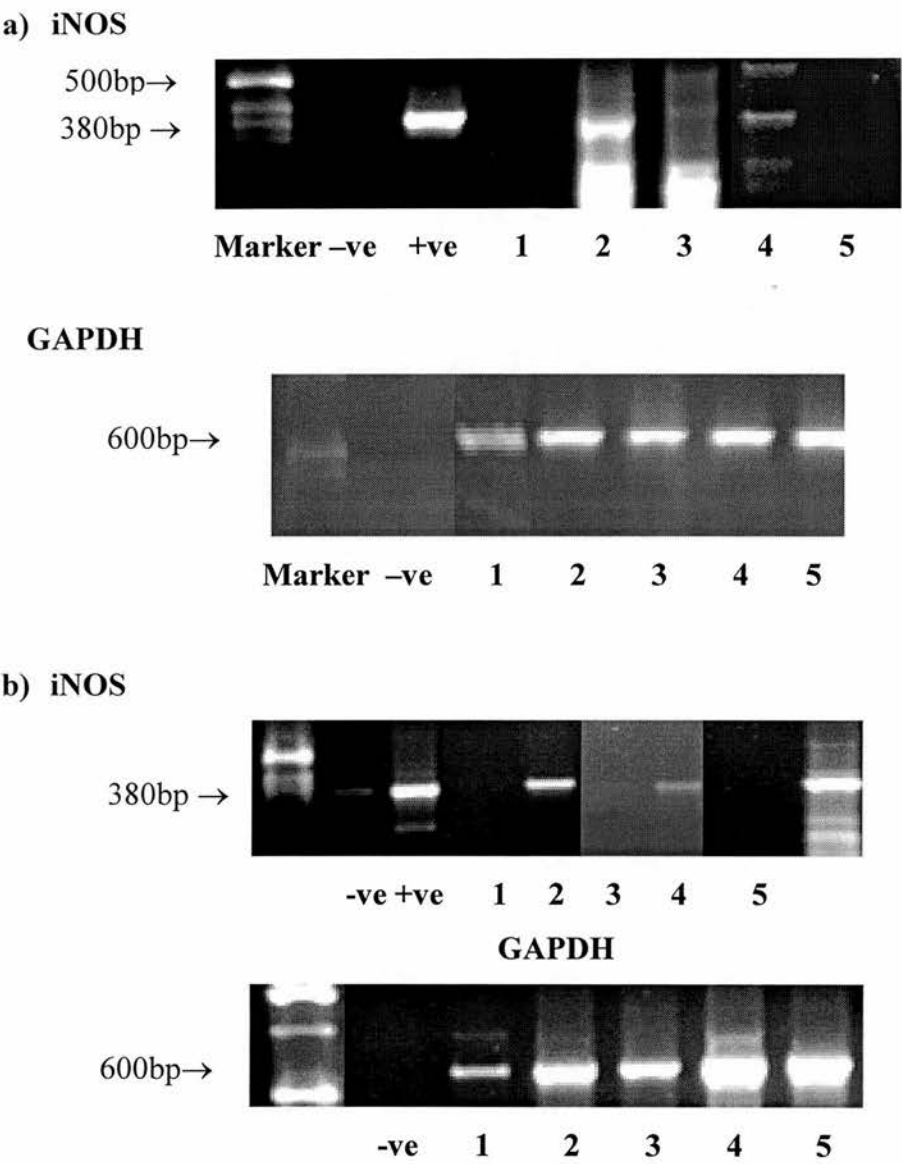


Figure 39: The effect of cytomix, IL1- β , TNF- α and IFN- γ on iNOS and GAPDH mRNA levels at 4 hours in a) A549 cells and b) 16HBE cells. -ve control, +ve control, **Lane 1**: control cells, **Lane 2**: cytomix, **Lane 3**: IL1- β , **Lane 4**: TNF- α , **Lane 5**: IFN- γ .

Figure 40 shows the ratio of iNOS to GAPDH following the above treatments.

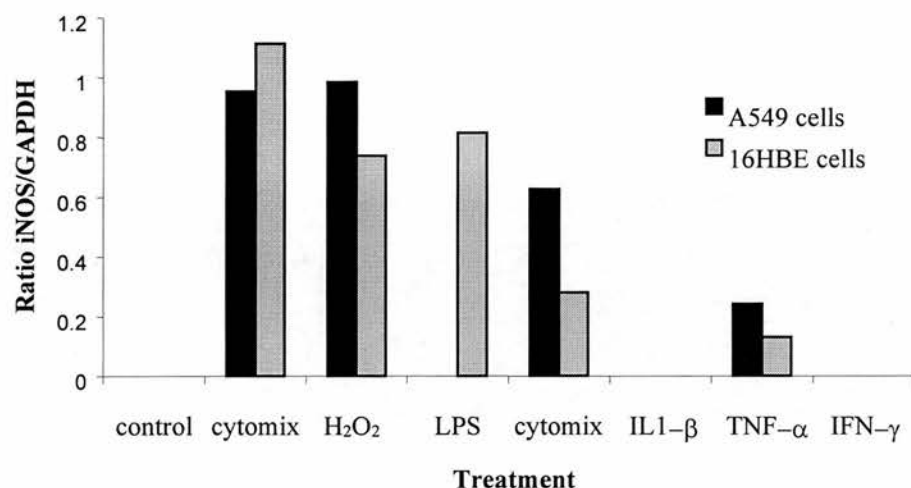


Figure 40: The ratio of iNOS mRNA to GAPDH in both A549 and 16HBE cells. following cytomix, H₂O₂, LPS, cytomix, IL1-β, TNF-α and IFN-γ treatment. Differences in the ratios observed do not represent differences in levels of NO[•] produced (n=1).

4.7 SUMMARY

- Cytomix, TNF-α and H₂O₂ induce NO[•] release, as measured by nitrite production in the cell medium, in both A549 and 16HBE cells following 24 hour treatments. IL1-β and IFN-γ have no effect on nitrite production.
- LPS induces NO[•] release in 16HBE cells but not in A549 cells following 24 hour treatment.
- iNOS induction can be observed between 1-16 hours (data not shown) and 4 hours was chosen as a time point to examine iNOS induction in both cell types.
- Cytomix, TNF-α and H₂O₂ induced iNOS in both A549 and 16HBE cells, LPS also induced iNOS in 16HBE cells.
- L-NMMA decreased NO[•] release in both A549 and 16HBE cells following cytomix and H₂O₂ treatment.
- GSH levels are decreased in epithelial cells concomitant with increased NO[•] release.

- GSH levels are decreased in cells pre-treated with L-NMMA followed by cytomix or H_2O_2 suggesting that the decrease in GSH is not due to NO^\cdot formation.

4.8 DISCUSSION

Nitric oxide can protect against cell damage and cytotoxicity caused by ROS by inhibiting ROS release from inflammatory cells such as neutrophils, by reacting with ROS to reduce their activity and by killing bacteria (Clancy et al., 1992; Granger and Kubes, 1996; Rubbo et al., 1994). iNOS is continuously expressed in airway epithelial cells in vivo, possibly to protect cells against general oxidant stress incurred in the lungs such as cigarette smoke, ozone and pollutants. However cells lose the ability to continually express iNOS upon culture (Nathan and Xie, 1994). Different cells are able to induce NOS following different stimuli, such as human hepatocytes which require LPS, $\text{IL1-}\beta$, $\text{TNF-}\alpha$, and $\text{IFN-}\gamma$ (Geller et al., 1993; Nussler et al., 1994), and human chondrocytes which only require $\text{IL1-}\beta$ (Maier et al., 1994). It has previously been reported (Robbins et al., 1994) that both A549 and primary human bronchial epithelial cells stimulated with cytomix ($\text{TNF-}\alpha$, $\text{IL1-}\beta$ and $\text{IFN-}\gamma$) release significant amounts of NO^\cdot at 24 hours, and express increased amounts of iNOS mRNA also at 24 hours. My results show that both A549 and 16HBE cells are able to produce significant amounts of nitrite in the cell medium on addition of cytomix for 24 hours. Both cell types also produce significant levels of nitrite at 24 hours with the addition of $\text{TNF-}\alpha$ alone, and 16HBE cells produce nitrite with the addition of LPS. However, $\text{IFN-}\gamma$ and $\text{IL1-}\beta$ have no effect on nitrite production in either cell type at 24 hours. It has also been reported (Adcock et al., 1994) that A549 cells release significant levels of NO^\cdot at 24 hours and have increased levels of iNOS mRNA at 2 hours on addition of the oxidant generator pyrogallol, suggesting that oxidants can also induce NO^\cdot . My results showed that both A549 and 16HBE cells were able to produce significant levels of nitrite at 24 hours on addition of a non-toxic dose of H_2O_2 , which served as a general oxidant stress. The dose of H_2O_2 was determined to be non-toxic in 2 ways. Cells were counted in the

experiments using trypan blue, and trypan blue exclusion by more than 95% of the cells indicates cells have maintained their integrity and the dose is non-toxic. The second measurement was made by Dr. B. Mulier, who measured the amount of lactate dehydrogenase (LDH) release from the cells into the medium. LDH release indicates that the cell membrane is no longer intact due to cell death, and the dose of H_2O_2 used herein did not cause cells to release LDH above control cell levels, indicating that the dose is non-toxic. Both these tests showed that the dose of H_2O_2 was non-toxic.

However, the addition of cytomix and H_2O_2 had no additive effect on NO^\cdot production in either cell type, nor did the addition of cytomix and LPS in 16HBE cells. This may be due to the assay since nitrite can readily become nitrate (which is not measured in the assay) or due to negative feedback. NO^\cdot has been shown to inhibit its own synthesis in a number of ways. Matthews *et al* (Matthews *et al.*, 1997) showed that NO^\cdot can inhibit NF- κB binding to DNA. NO^\cdot donors were used and were shown to inhibit binding by S-nitrosylation of redox sensitive cysteine residues on the p50 subunit, the necessary subunit for DNA binding. Peng *et al* (Peng *et al.*, 1995) also showed by immunoprecipitation studies and NO^\cdot donors that NO^\cdot stabilised the inhibitory subunit of NF- κB , I κB α by preventing its degradation from NF- κB . NO^\cdot was also able to increase mRNA levels for I $\kappa\text{B}\alpha$, and transfection experiments suggested that NO^\cdot can transcriptionally induce I κB α . Evidence therefore suggests that cellular NO^\cdot can provide a control mechanism for modulating the expression of NF- κB responsive genes, such as the induction of further iNOS, hence it provides a negative feedback mechanism for itself.

The induction of iNOS has been previously shown to occur between 1 and 24 hours in various cell types (Adcock *et al.*, 1994; Assreuy *et al.*, 1993; Chesrown *et al.*, 1994; Robbins *et al.*, 1994), and my own data shows that iNOS mRNA can be observed clearly at 4 hours when measured by RT-PCR in both cell types. iNOS mRNA was increased in both A549 and 16HBE cells following cytomix, H_2O_2 and TNF- α . iNOS mRNA was also increased in 16HBE cells following LPS. Neither IL1- β or IFN- γ had any effect on iNOS induction in either cell type.

The glutathione redox system is important in cell protection during oxidant stress, preventing oxidant-induced damage such as cell leakage, and maintaining membrane integrity (Pietarinen et al., 1995). There are several reports indicating that both NO[•] and GSH may regulate each other. Phelps *et al* (Phelps et al., 1995) reported that the induction of NO[•] in bovine lung endothelial cells by TNF- α , resulted in decreased GSH levels at 6 hours via a protein kinase C pathway, due to the formation of ONOO⁻ and that this also results in an altered GSH:GSSG ratio. Both L-NMMA and SOD, which prevent NO[•] synthesis or superoxide formation respectively, could block these effects suggesting that either NO[•] formation, oxidants or ONOO⁻ are necessary for the decrease in GSH. In contrast to Phelps *et al* (Phelps et al., 1995), White *et al* (White et al., 1995) showed that NO[•] donors increased GSH in RFL6 cells (rat lung fibroblasts), possibly by the upregulation of GSH synthesis. Inhibition of cytokine-induced NO[•] (Kuo and Abe, 1996a) in rat hepatocytes also resulted in decreased GSH levels, which were restored by NO[•] donors. These reports suggest that NO[•] may be involved in the regulation of GSH synthesis and this is a possible mechanism by which NO[•] could protect against oxidant-induced injury. Furthermore, reduced GSH has been shown to be critical for the induction of iNOS (Duval et al., 1995). Inhibition of GSH de novo synthesis by buthionine sulfoximine (BSO) or inhibition of the reduction of GSSG by Bis-2 chloroethyl nitosourea (BCNU) abolishes or reduces NOS induction by TNF- α (Duval et al., 1995). The enzyme iNOS has several co-factors, one of which, is GSH (Steuhr et al., 1990), and GSH is therefore considered necessary for enzyme activation. Inhibition of GSH synthesis or reduction may therefore prevent NO[•] production.

Since the hypothesis of this thesis is that intracellular GSH levels may be involved in NO induction, I measured the levels of intracellular GSH following cytokine and oxidant treatments. My results show that NO[•] production at 24 hours is concomitant with a decrease in intracellular GSH, suggesting that in this system NO[•] does not up-regulate GSH synthesis. It was possible, however, that the decrease in intracellular GSH was due to NO[•] production. In order to determine if NO[•] induction caused a decrease in GSH, the NO[•] inhibitor L-NMMA was used followed by cytomix or H₂O₂. L-NMMA prevented cytomix and H₂O₂ induced NO[•] production but did not

prevent the decrease in GSH observed with cytomix or H_2O_2 alone. This suggests that the decrease in intracellular GSH is due, not to NO^\cdot production, but due to the cytomix and H_2O_2 themselves.

$\text{TNF-}\alpha$ causes an increase in both ROS and NO^\cdot production, and iNOS induction by $\text{TNF-}\alpha$ can be inhibited by the addition of the mitochondria complex I inhibitor rotenone in human hepatocytes (Duval et al., 1995). Rotenone inhibits the reduction of oxygen to water by inhibiting electron flow in the electron transport chain, and thus preventing the release of superoxide anions from the mitochondria. The use of rotenone in preventing $\text{TNF-}\alpha$ induced iNOS activation provides evidence that $\text{TNF-}\alpha$ works via the mitochondria, releasing ROS and suggests that oxidants are necessary for iNOS induction. Exposure of the cell to extracellular ROS, generated by the xanthine/xanthine oxidase system, also increases NOS activity, further supporting the hypothesis that ROS are involved in iNOS induction (Duval et al., 1995). It has also been shown that $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$ (Kuo et al., 1996b) act synergistically in mouse hepatocytes, and that $\text{IFN-}\gamma$ increases both $\text{TNF-}\alpha$ induced iNOS activation and the generation of ROS. $\text{IFN-}\gamma$ also potentiates the $\text{TNF-}\alpha$ induced effects on GSH, causing a depletion in GSH and a flux of GSSG out of the cells. The synergistic effect is thought to be due to $\text{IFN-}\gamma$ increasing $\text{TNF-}\alpha$ receptors. Thus it is likely that ROS are involved in the decrease in GSH within the cell and it is possible that both ROS and GSH are involved in NO^\cdot induction.

As mentioned previously decreasing GSH with BSO and BCNU (Duval et al., 1995) reduces NOS induction but it has also been shown that antioxidants decrease iNOS induction (Hecker et al., 1996a; Hecker et al., 1996b). These reports (Duval et al., 1995; Hecker et al., 1996a; Hecker et al., 1996b), although appearing to show opposite results suggest that GSH may be involved in NOS induction. ROS have been shown to decrease GSH (Duval et al., 1995), which is oxidized to GSSG, suggesting there may be a complex link between oxidants, antioxidants and GSH in the induction of iNOS. It has also been shown in J774 cells, (a murine macrophage cell line), (Hothersall et al., 1997) that the addition of LPS and $\text{IFN-}\gamma$ causes a 45% decrease in GSH at 48 hours and a decrease in the GSH:GSSG ratio from 12:1 to 2:1,

and that this change is not due to the efflux of GSH. This suggests that there is either a decrease in the de novo synthesis of GSH or there is an increase in its utilisation and that this change may be important in iNOS induction.

There is conflicting evidence, however, about the decrease in GSH following iNOS induction. Some reports suggest that the decrease in GSH following iNOS induction is dependent on the production of NO \cdot , since the decrease can be prevented by addition of the inhibitor L-NMMA (Hothersall et al., 1997). A decrease in intracellular GSH with compounds such as BSO also decreases the output of NO \cdot following activation suggesting GSH is involved in NO \cdot induction (Hothersall et al., 1997). In contrast, Kuo *et al.*, (Kuo et al., 1996b) suggest that TNF- α and IFN- γ cause an increase in oxidant stress within the cells, resulting in altered GSH levels and an efflux of GSSG, independent of whether NO \cdot is produced or not. In these studies the TNF- α and IFN- γ induced GSSG efflux was not dependant on the stimulation of NOS, as shown by the studies using the inhibitor L-NMMA, and also, the GSSG efflux occurred in the first 4 hours, a time point at which significant levels of NOS activation were not observed (Kuo et al., 1996b). This report is similar to my own results, where a decrease in GSH is observed in both A549 and 16HBE cells following cytomix or H $_2$ O $_2$ stimulation but the addition of L-NMMA followed by cytomix or H $_2$ O $_2$ stimulation does not prevent the decrease in GSH. This suggests that the decrease is due to an increased oxidant burden, causing an increase in the oxidation of GSH to GSSG which is subsequently removed from the cells. It is also possible that an increase in the GSSG to GSH ratio is important in the induction of iNOS. NO \cdot itself did not cause a decrease in GSH in my system at 24 hours but it is possible that at later time points, NO \cdot may cause further decreases in GSH. NO \cdot can rapidly bind to thiol receptor molecules such as GSH, forming nitrosothiols which are not detected in the Griess assay, and this is a possible cause for the NO \cdot -dependant decrease in GSH observed in other systems. NO \cdot also rapidly reacts with superoxide anions, such as those produced by the mitochondria, to form peroxynitrite, which is a potent oxidant, causing a decrease in GSH and is thus another possible mechanism for the decrease in GSH. However, since the decrease in GSH occurs with cytomix and H $_2$ O $_2$ stimulation, independently of NO \cdot release, it can

be assumed that the decrease in GSH observed in my studies is not due to the formation of GSNO or due to peroxynitrite formation, but is due to the oxidant burst induced by TNF- α or oxidant stress induced by H₂O₂.

Thus there is conflicting evidence concerning the role of NO \cdot in GSH regulation and the importance of GSH in NO \cdot induction. My data support the hypothesis that GSH may be important in NO \cdot induction but that the involvement of GSH may be indirect. It is possible that superoxides are important in induction of NO \cdot , and the presence of GSH is important in maintaining an antioxidant state within the cell to prevent NO \cdot induction. Or, it may be that the ratio of GSH:GSSG is important since GSH levels are decreased in NO \cdot induced cells and a decrease in GSH could result from the oxidation of GSH to GSSG. Manipulation of GSH levels and its effect on NO \cdot induction are studied in more detail in chapter 5.

Chapter 5 The Effect of Manipulating GSH Levels on NO[•] Induction and Release In Both A549 and 16HBE Cells.

5.1 INTRODUCTION

GSH is an important antioxidant in the lungs, it both reacts with ROS and acts as a substrate for glutathione peroxidase (GPx) as part of the GSH redox enzyme system (Cantin and Begin, 1991). The redox balance of the cell is considered to be important in lung inflammation, since changes in the redox balance may be critical in modulating molecular events in the cell. These include initiating a cascade of events such as the activation of NF- κ B, which, in turn, regulates gene expression for many inflammatory mediators including NO[•] (Adcock et al., 1994). The addition of antioxidants to lung cells can protect against oxidant damage by either scavenging ROS or by increasing both intra and extra-cellular GSH levels (Mulier et al., 1998).

The previous chapter reports that NO[•] and iNOS can be induced by a variety of stimuli in both A549 and 16HBE cells and that this induction is concomitant with a decrease in GSH levels, independently of the formation of NO[•]. This suggests that GSH may be involved, either directly or indirectly via ROS, in the induction of NO[•].

This chapter examines the effects of manipulating GSH levels before cells are stimulated in order to determine the effect of modulating GSH levels on NO[•] levels.

5.2 MANIPULATION OF GSH LEVELS.

GSH was manipulated by:

- Decreasing GSH using the irreversible inhibitor of γ -glutamylcysteine synthase (γ -GCS), buthionine sulfoximine (BSO). γ -GCS is the rate-limiting enzyme involved in GSH synthesis.
- Increasing GSH levels using 4 different thiol compounds:

- ◆ N-acetylcysteine (NAC)
- ◆ N-acetylcysteine lysinate (NAL)
- ◆ Glutathione (GSH)
- ◆ Glutathione monoethylester (GSHMEE)

These 4 compounds all increase intracellular GSH levels but in slightly different ways.

NAC and NAL are both precursors of the amino acid cysteine and are thought to work by either intracellular or extracellular deacetylation to release cysteine (Phelps et al., 1992). Cysteine, which can be a rate limiting amino acid in GSH synthesis, is then used for denovo synthesis of GSH in the cell. GSH is thought to increase GSH by either being degraded extracellularly by the membrane bound γ -glutamyl transpeptidase, and its respective amino acids taken up for denovo synthesis of GSH, or by reducing extracellular cystine to cysteine, which is then taken into the cell for denovo synthesis (Deneke et al., 1995). GSHMEE, in contrast to GSH, effectively crosses the membrane due to its electrophilic surface and is converted to intracellular GSH by esterases, which remove the ester group (Anderson et al., 1985).

5.3 THE EFFECT OF DEPLETING INTRACELLULAR GSH WITH BSO ON NO PRODUCTION.

A concentration of 50 μ M BSO was chosen for these studies since previous studies have used this concentration and it has been found to be effective in decreasing GSH without causing toxicity (Beasley et al., 1985; Poot et al., 1995) and trypan blue exclusion was used to ensure this level was not toxic in either the A549 or 16HBE cells. Cells were treated at 70% confluency with 50 μ M BSO and BSO plus cytomix in 2% medium for 24 hours. GSH levels are depleted in both A549 and 16HBE cells by approximately 90% compared to control in both cells treated with BSO and BSO with cytomix (Figures 41 and 42). Cytomix also causes depletion of GSH (as

previously shown in Figures 28 and 29). However there was no significant difference between cells treated with BSO alone and BSO plus cytomix. BSO also significantly decreased GSH compared to cytomix alone.

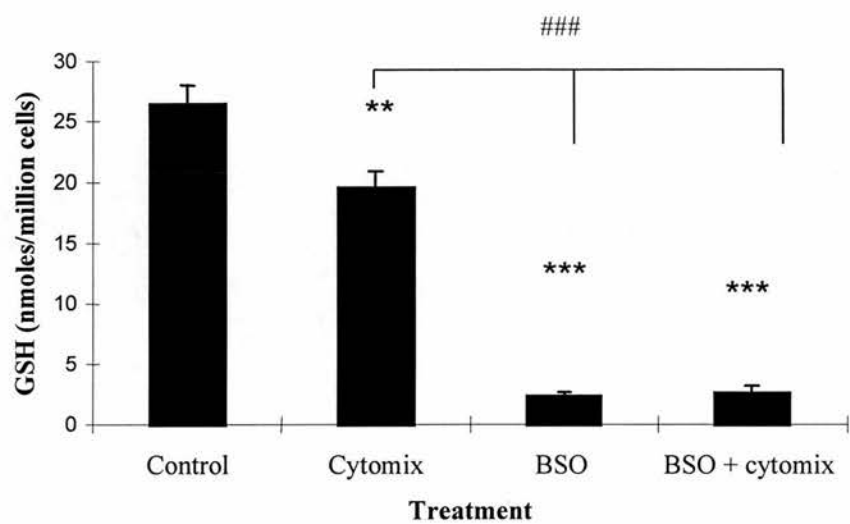


Figure 41: The effect of BSO on intracellular GSH levels in A549 cells at 24hrs. Histograms represent means and bars SEMs. (**p<0.01, ***p<0.001 compared to control, ###p<0.001, compared to cytomix).

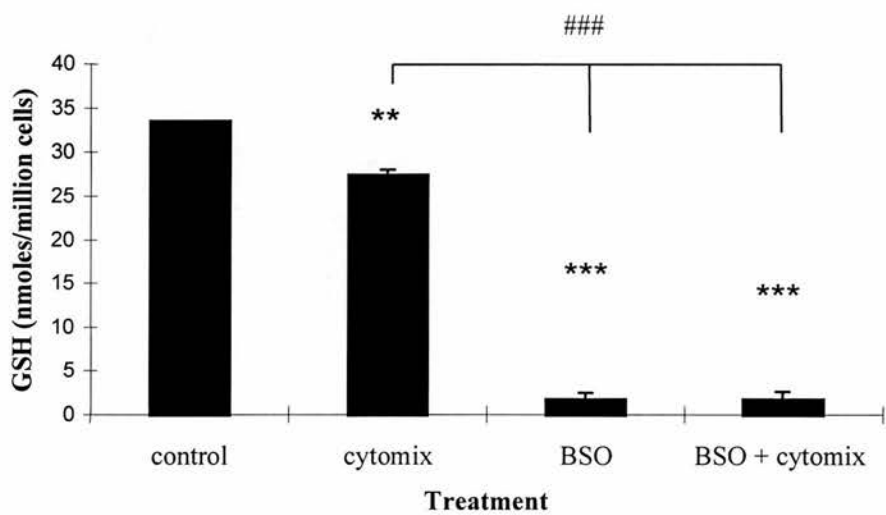


Figure 42: The effect of BSO on intracellular GSH levels in 16HBE cells at 24hrs. Histograms represent means and bars SEMs. (**p<0.01, ***p<0.001 compared to control, ###p<0.001, compared to cytomix).

Figures 43 and 44 show the effect of BSO on nitrite levels in both A549 and 16HBE cells at 24 hours. BSO produced a significant increase in nitrite levels in both cell types at 24 hours. Although cells treated with BSO plus cytomix have significantly higher levels of nitrite compared to cytomix and BSO alone in A549 cells, there was no significant difference between BSO plus cytomix and BSO or cytomix alone. Thus there is no additive effect of co-treating cells with both BSO and cytomix.

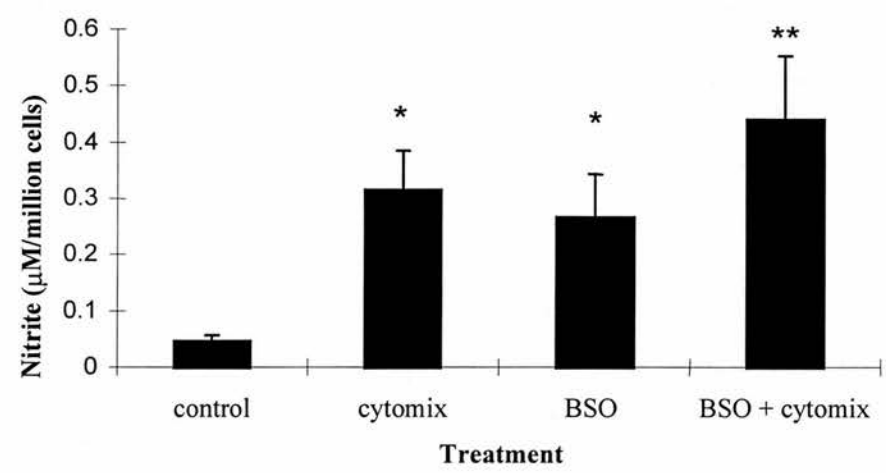


Figure 43: The effect of BSO on nitrite production in A549 cells at 24 hours. Histograms represent means and bars SEMs. (* $p<0.05$, ** $p<0.01$, compared to control).

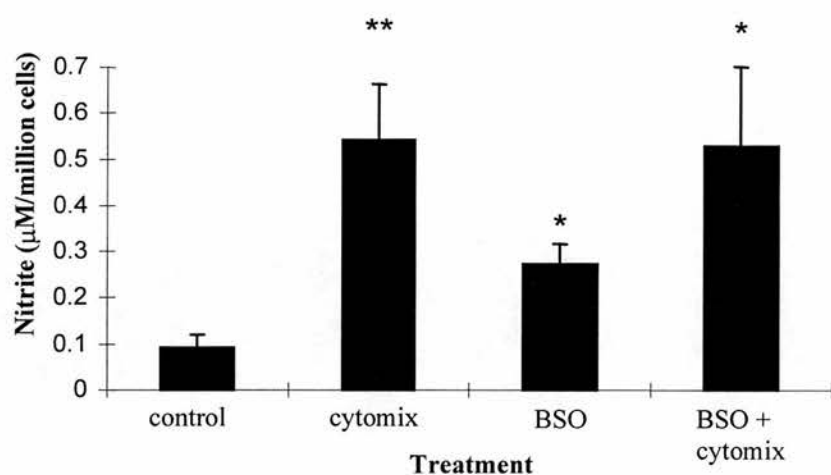


Figure 44: The effect of BSO on nitrite production in 16HBE cells at 24 hours. Histograms represent means and bars SEMs. (* $p < 0.05$, ** $p < 0.01$, compared to control).

Since cells were co-treated with cytomix and BSO the effect of BSO + cytomix on cells may not have been observed because cytomix may already have induced NO before GSH levels were decreased. Cells were therefore pre-treated with BSO for 16 hours at which point GSH levels were reduced to approximately 90% compared to control cells (figure 45).

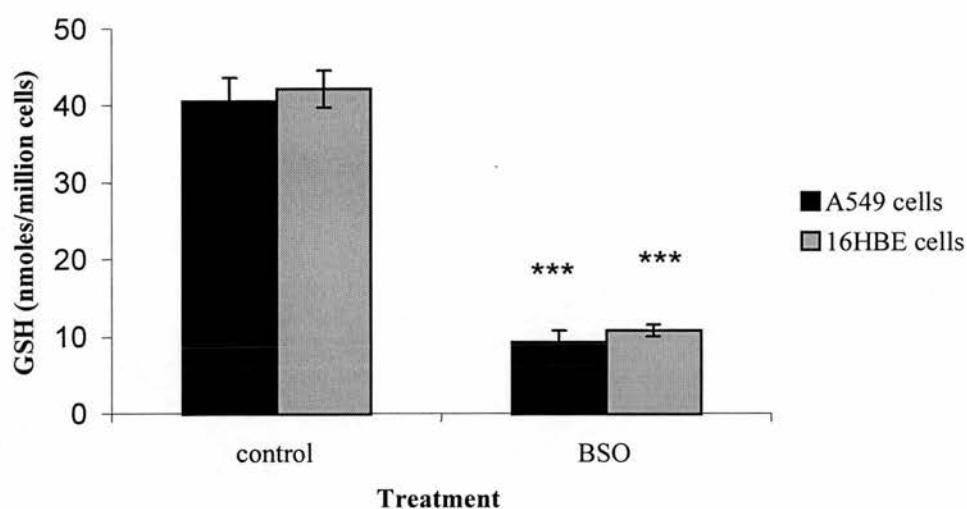


Figure 45: The effect of BSO on GSH levels in both A549 and 16HBE cells at 16 hours. Histograms represent means and bars SEMs. (***) $p < 0.001$ compared to the relevant control).

Cells were then treated with cytomix for 24 hours to determine if decreased GSH levels had any effect on NO^\bullet induction. Figures 46 and 47 show the effects of pre-treating cells for 16 hours with BSO followed by cytomix for 24 hours on nitrite levels in both A549 and 16HBE cells. Decreasing GSH levels had no effect on cytomix induced NO^\bullet release.

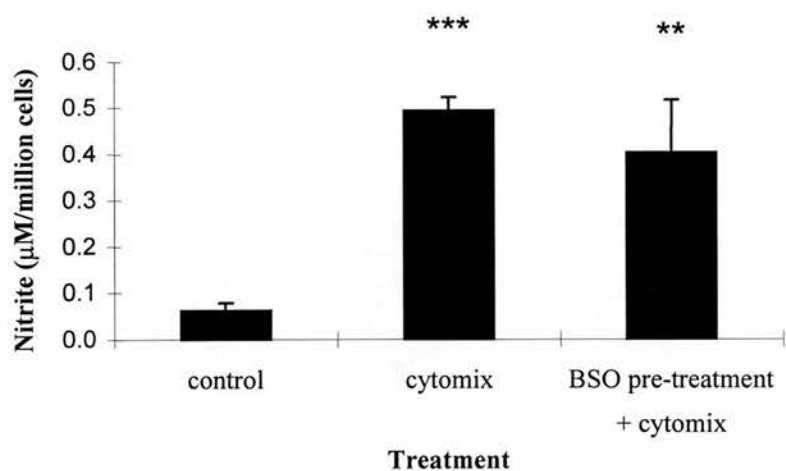


Figure 46: The effect of pre-treating cells for 16 hours with BSO followed by cytomix for 24 hours on nitrite levels in A549 cells. Histograms represent means and bars SEMs. (**p<0.01 compared to control).

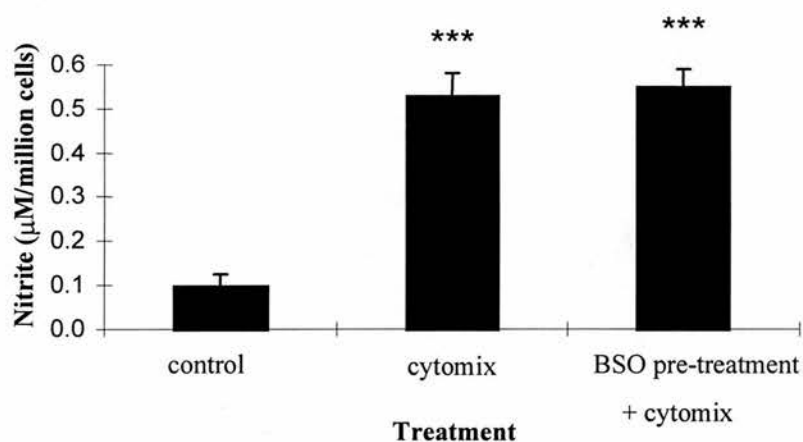


Figure 47: The effect of pre-treating cells for 16 hours with BSO followed by cytomix for 24 hours on nitrite levels in 16HBE cells. Histograms represent means and bars SEMs. (**p<0.01 compared to control).

5.4 THE EFFECT OF INCREASING GSH LEVELS WITH THIOL COMPOUNDS ON NO[•] RELEASE.

Since decreasing GSH levels resulted in increased NO[•] release I hypothesised that increasing GSH levels with antioxidants would prevent NO[•] release i.e. antioxidants may have a protective effect. Cells were treated with the four thiol compounds already mentioned, NAC, NAL, GSH, and GSHMEE to determine if they could protect prevent NO[•] induction following cytomix or H₂O₂ treatment. Cells were treated with NAC, NAL, GSH and GSHMEE all at 5mM in 2% medium and GSH levels were measured over a period of 24 hours (Figures 47 and 48). An increase in GSH was observed in both cells between 4 and 6 hours, with a highly significant increase (50-150%) occurring at 4 hours, with all four thiol compounds.

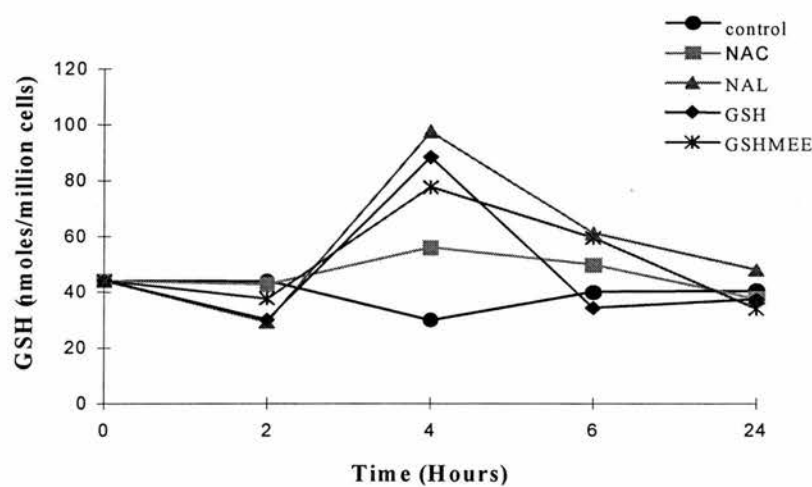


Figure 47: The effect of the 4 thiol compounds on intracellular GSH levels in A549 cells over 24hrs. Symbols represent means, SEMs are omitted to add clarity.

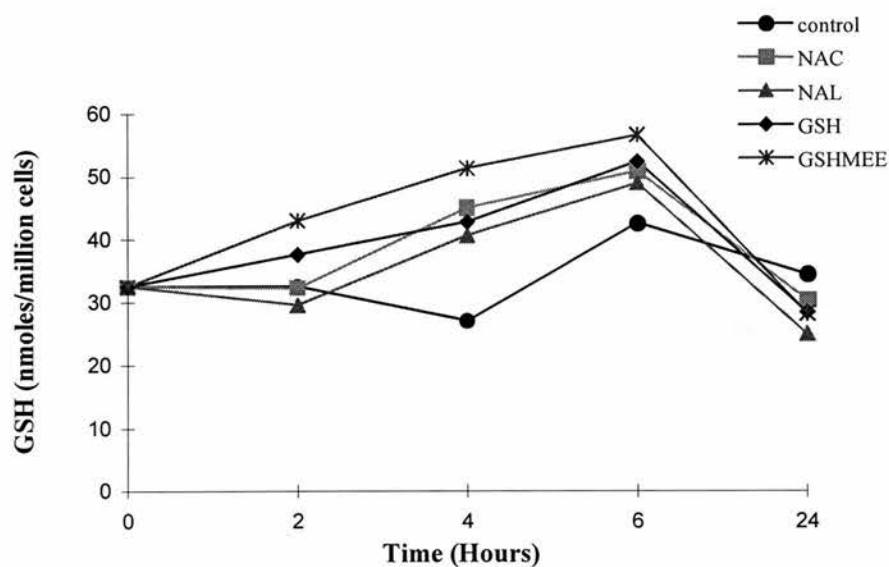


Figure 48: The effect of the 4 thiol compounds on intracellular GSH levels in 16HBE cells over 24hrs. Points represent means, SEMs are omitted to add clarity.

The significant values of increasing GSH levels at 4 hours in both cell types with the 4 thiol compounds are shown in figures 49 and 50.

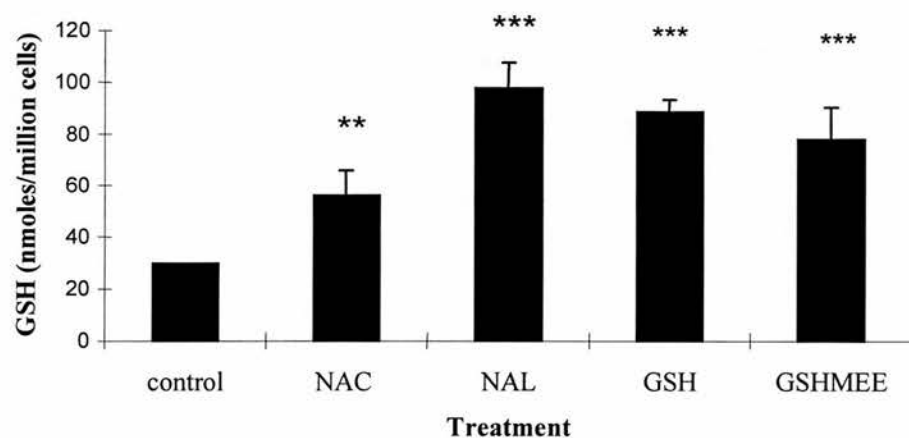


Figure 49: The effect of the 4 thiol compounds, NAC, NAL, GSH and GSHMEE on GSH levels at 4hrs in A549 cells. Histograms represent means bars SEMs (**p<0.01, ***p<0.001 compared to control).

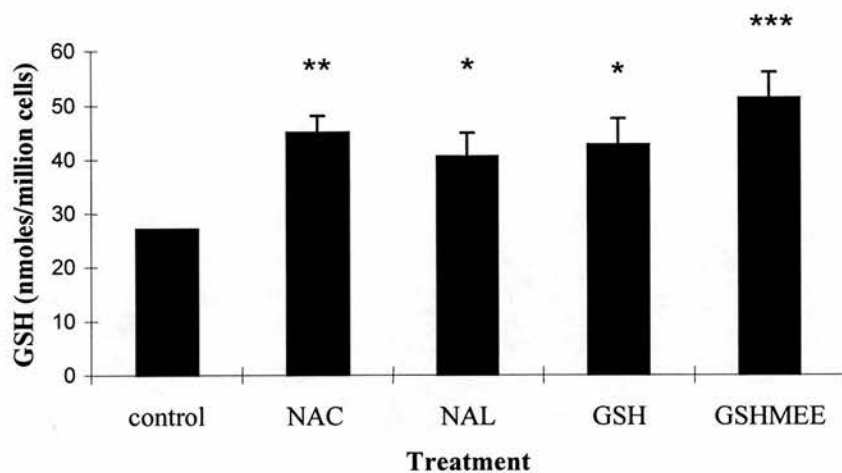


Figure 50: The effect of the 4 thiol compounds, NAC, NAL, GSH and GSHMEE on GSH levels at 4hrs in 16HBE cells. Histograms represent means bars SEMs (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to control).

I have previously shown (section 4.1) that NO^\cdot induction by cytomix, as measured by nitrite production, does not occur until 24 hours, the effect of the 4 thiol compounds on nitrite production was examined at 4 hours, the time at which GSH levels are increased. The 4 thiol compounds had no effect on nitrite production at 4 hours when GSH is increased (figures 51 and 52).

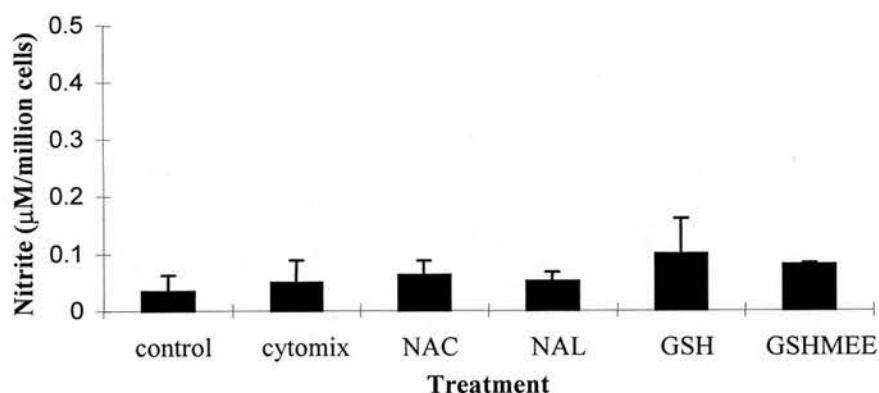


Figure 51: The effect of thiol compounds on nitrite production in A549 cells at 4hrs. Histograms represent means and bars SEMs.

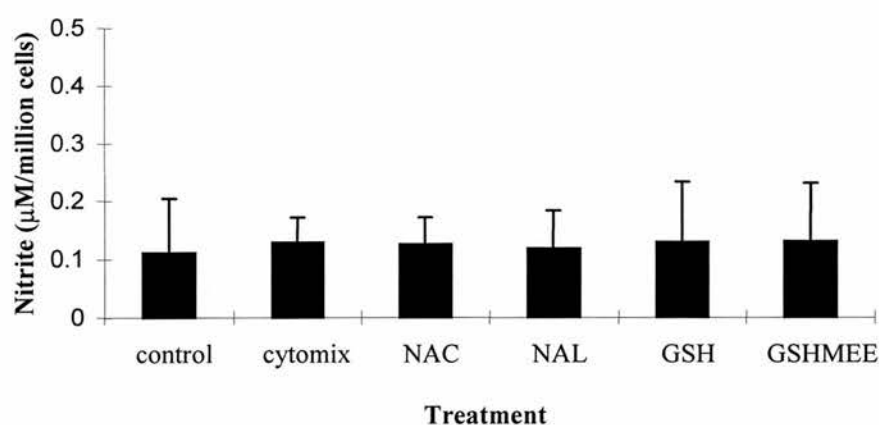


Figure 52: The effect of thiol compounds on nitrite production in 16HBE cells at 4hrs. Histograms represent means and bars SEMs.

Nitrite levels were also measured at 24 hours in both cell types following thiol treatment (Figures 53 and 54) which showed that the four thiol compounds had no effect on nitrite production over 24 hours. GSH levels have returned to control values at this point but increasing GSH levels at 4 hours did not itself have any effect on NO[•] release from cells compared to cytomix.

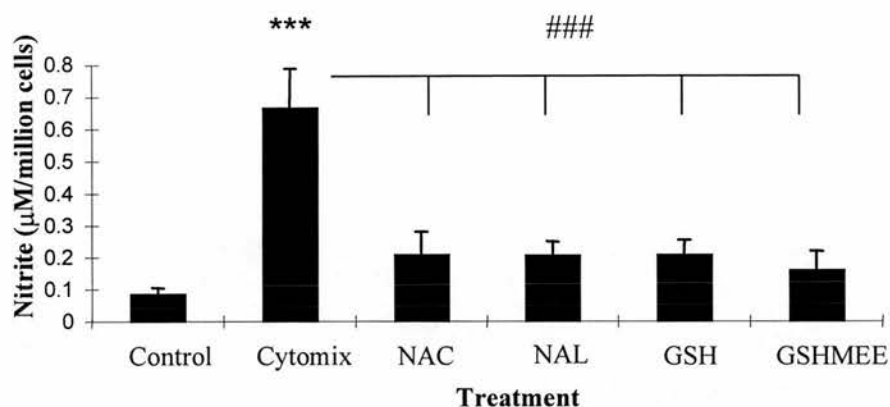


Figure 53: The effect of thiol compounds on nitrite prodction in A549 cells at 24hrs. Histograms represent means and bars SEMs. (***) $p < 0.001$, compared to control, ### $p < 0.001$, compared to cytomix).

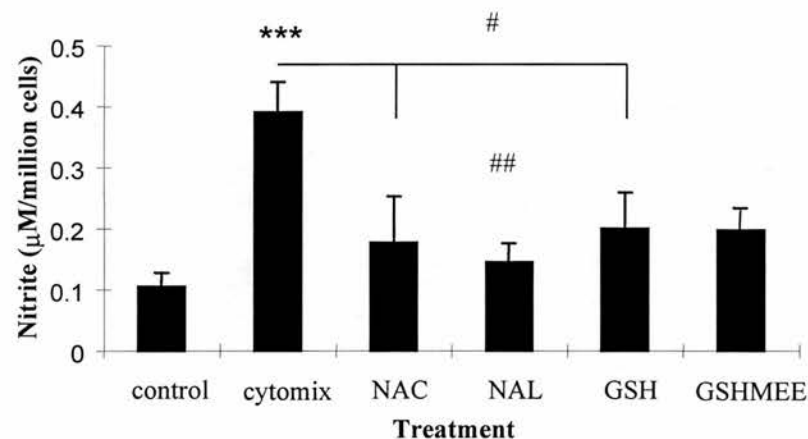


Figure 54: The effect of thiol compounds on nitrite production in 16HBE cells at 24hrs. Histograms represent means and bars SEMs. (***) $p < 0.001$, compared to control, # $p < 0.05$, ## $p < 0.01$, compared to cytomix).

5.5 THE EFFECT OF PRE-TREATING CELLS WITH THIOL COMPOUNDS ON CYTOMIX AND H₂O₂ INDUCED NO[•] RELEASE.

Cells were pre-treated with the four thiol compounds for 4 hours, washed in PBS three times (to ensure the antioxidants did not affect the cytomix), and treated with cytomix for 24 hours in order to determine if increased intracellular GSH levels could inhibit cytomix induced NO[•] release. Figures 55 and 56 show the effect of pre-treating cells with thiol compounds in both A549 and 16HBE cells followed by cytomix for 24 hours. Increased GSH levels did not prevent cytomix induced NO[•] release.

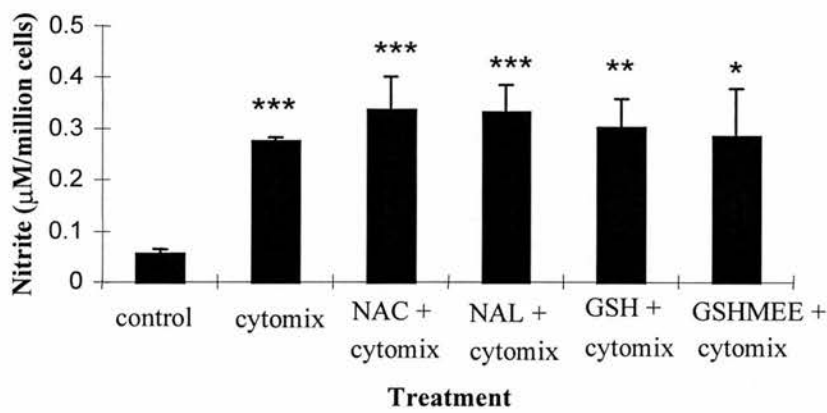


Figure 55: The effect of pre-treating A549 cells with 4 thiol compounds for 4hrs followed by cytomix for 24hrs. Histograms represent means and bars SEMs. (*p<0.05, **p<0.01, ***p<0.001, compared to control).

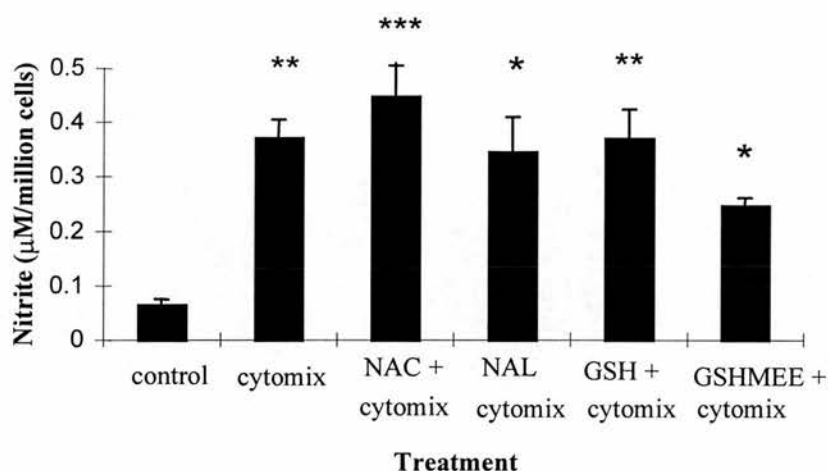


Figure 56: The effect of pre-treating A549 cells with 4 thiol compounds for 4 hrs followed by cytomix for 24hrs. Histograms represent means and bars SEMs. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, compared to control).

Cells were then pre-treated with the four thiol compounds for 4 hours followed by H_2O_2 for 24 hours to determine if increasing GSH levels could prevent oxidant induced NO release. Cells were again washed 3 times in PBS to ensure the thiol compounds did not scavenge the H_2O_2 . Figures 57 and 58 show that increasing intracellular GSH levels in both A549 and 16HBE cells significantly decreased H_2O_2 induced NO release by at least 50%.

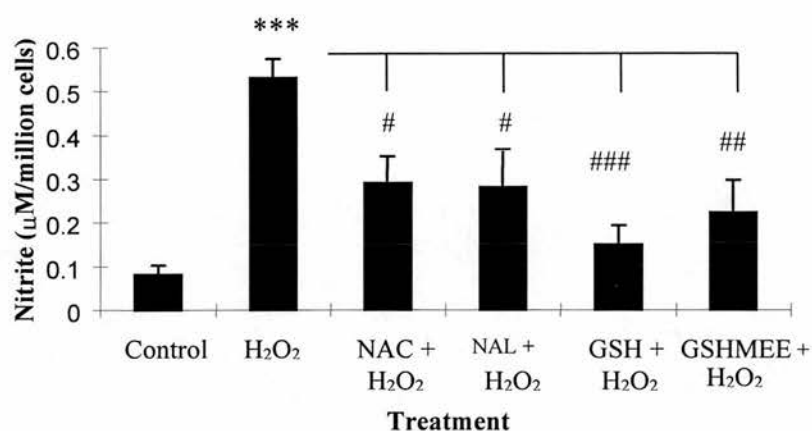


Figure 57: The effect of pre-treating A549 cells with thiol compounds for 4 hrs followed by H₂O₂ for 24hrs. Histograms represent means and bars SEMs. (***) $p < 0.001$ compared to control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, compared to H₂O₂).

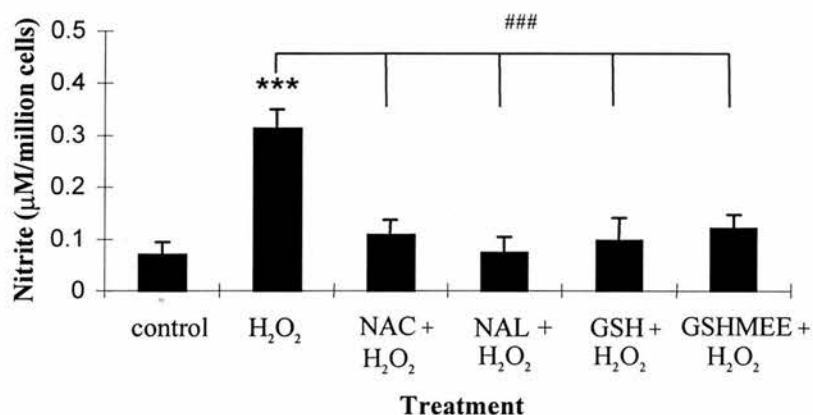
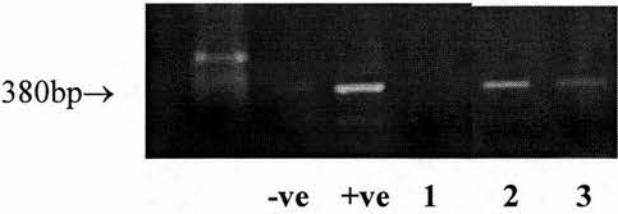


Figure 58: The effect of pre-treating 16HBE cells with thiol compounds for 4 hrs followed by H₂O₂ for 24hrs. Histograms represent means and bars SEMs. (***) $p < 0.001$ compared to control, ### $p < 0.001$, compared to H₂O₂).

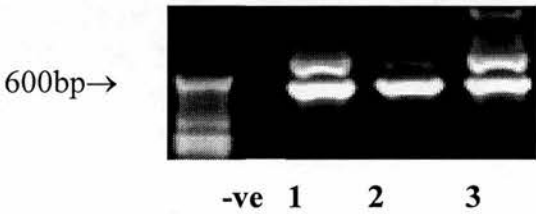
5.6 PCR RESULTS

Since BSO resulted in increased NO[•] levels, iNOS and GAPDH mRNA levels were measured in both A549 and 16HBE cells. iNOS mRNA was not observed at the 4 hour time-point used for all other measurements (data not shown), in A549 cells and therefore a later time-point of 6 hours was used. Figure 60 shows the effects of BSO at 6 hours in A549 cells and 4 hours in 16HBE cells. Figure 61 shows the ratio of iNOS to GAPDH.

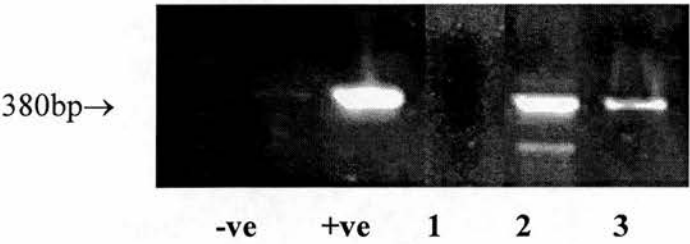
a) iNOS



GAPDH



b) iNOS



GAPDH

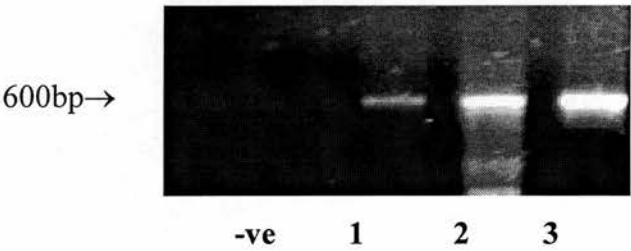


Figure 60: The effect of BSO on iNOS and GAPDH mRNA in both A549 at 6 hours and 16HBE cells at 4 hours. -ve control, +ve control, **Lane 1**: control, **Lane 2**: cytomix, **Lane 3**: BSO.

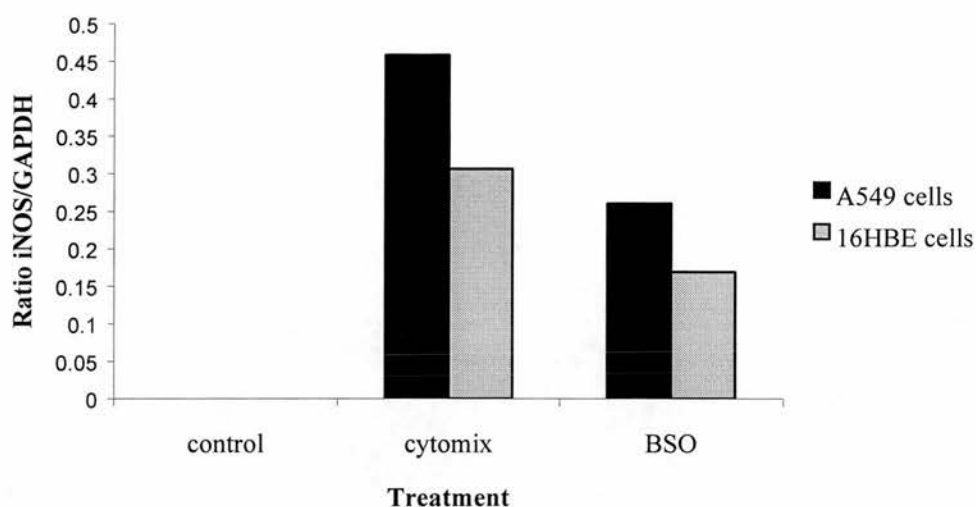
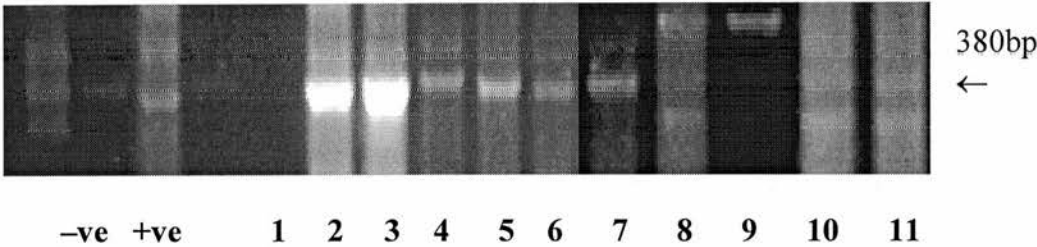


Figure 61: The effect of treating both A549 (6hrs) and 16HBE cells (4hrs) with BSO at 6 hours. Differences in the ratios observed do not represent differences in levels of $\text{NO}^{\cdot-}$ produced (n=1).

iNOS and GAPDH mRNA were measured in both A549 and 16HBE cells pre-treated with the 4 thiol compounds, which had no effect on iNOS induction themselves (data not shown), followed by either cytomix or H_2O_2 . Figure 62 and 63 show the effect of pre-treatment in both the cells types on iNOS mRNA. The thiol compounds have no effect on cytomix induced iNOS but reduce H_2O_2 induced iNOS in both cells types. Figure 64 shows the ratio of iNOS to GAPDH following these treatments.

INOS



GAPDH

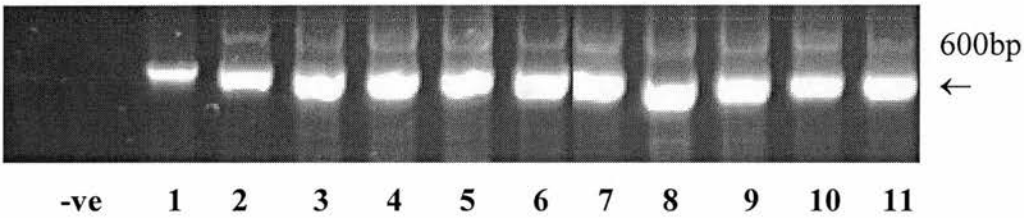


Figure 62: The effect of pre-treating A549 cells with the 4 thiol compounds, NAC, NAL, GSH and GSHMEE followed by cytomix and H₂O₂. -ve, +ve control, **Lane 1:** control, **Lane 2:** cytomix, **Lane 3:** NAC + cytomix, **Lane 4:** NAL + cytomix, **Lane 5:** GSH + cytomix, **Lane 6:** GSHMEE + cytomix, **Lane 7:** H₂O₂, **Lane 8:** NAC + H₂O₂, **Lane 9:** NAL + H₂O₂, **Lane 10:** GSH + H₂O₂, **Lane 11** GSHMEE + H₂O₂.

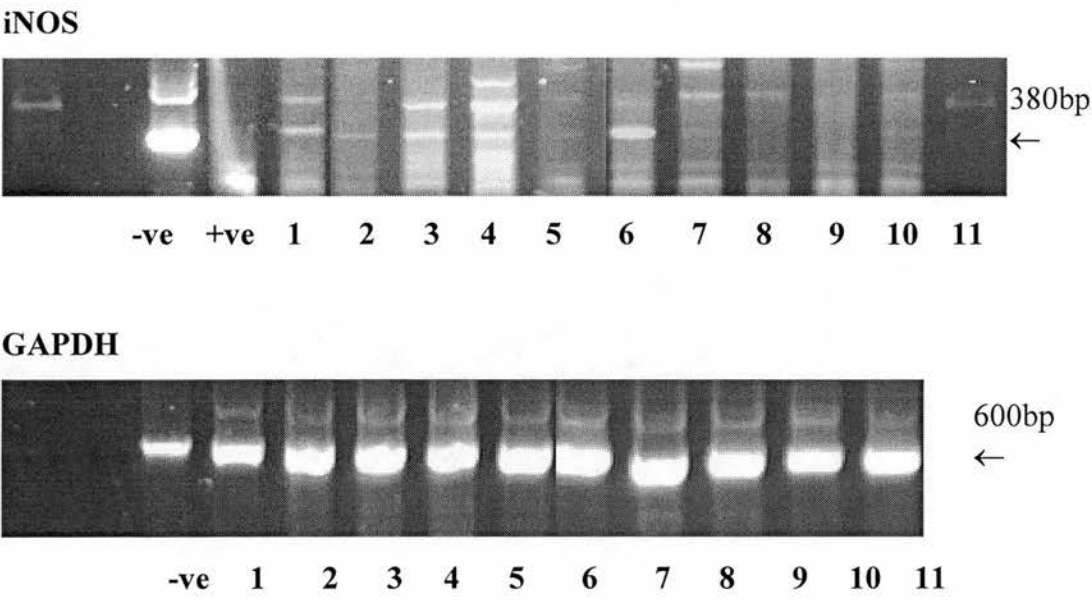


Figure 63: The effect of pre-treating 16HBE cells with the 4 thiol compounds, NAC, NAL, GSH and GSHMEE followed by cytomix and H₂O₂. -ve, +ve control, **Lane 1:** control, **Lane 2:** cytomix, **Lane 3:** NAC + cytomix, **Lane 4:** NAL + cytomix, **Lane 5:** GSH + cytomix, **Lane 6:** GSHMEE + cytomix, **Lane 7:** H₂O₂, **Lane 8:** NAC + H₂O₂, **Lane 9:** NAL + H₂O₂, **Lane 10:** GSH + H₂O₂, **Lane 11:** GSHMEE + H₂O₂.

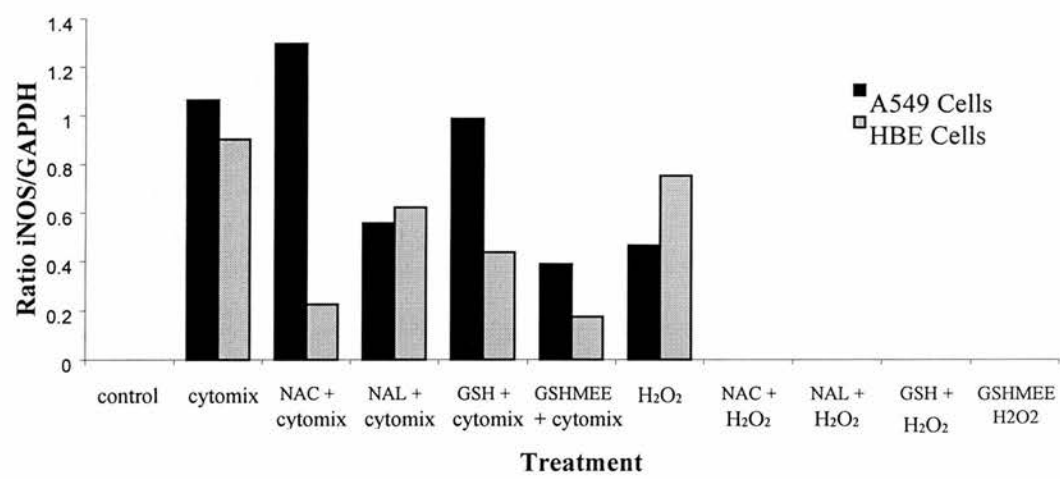


Figure 64: The effect of pre-treating both A549 and 16HBE cells with the 4 thiol compounds followed by cytomix and H₂O₂. Differences in the ratios observed are not meant to represent differences in levels of NO[•] produced (n=1).

5.7 SUMMARY

- Treatment of both A549 and 16HBE cells with BSO resulted in decreased GSH levels at 24 hours.
- This treatment also resulted in an increase in NO[•] and iNOS mRNA in both A549 and 16HBE cells.
- Pre-treatment of both cell types with BSO followed by cytomix did not have any additional effect on NO[•] release.
- The four thiol compounds; NAC, NAL, GSH and GSHMEE increased GSH levels significantly in both A549 and 16HBE cells at 4 hours.
- Pre-treatment of both cell types with the 4 thiol compounds followed by cytomix had no effect on NO[•] release or iNOS induction.
- Pre-treatment of both cell types with the 4 thiol compounds followed by H₂O₂ produced a significant decrease in both NO[•] release and iNOS compared to H₂O₂ alone.

5.8 DISCUSSION

Reduced glutathione (GSH) is a key intracellular antioxidant, protecting cells against oxidant-mediated injury, in particular against oxidants produced during inflammation. There is increasing evidence that GSH is also involved in the induction of iNOS, (Hothersall et al., 1997) which causes the release of NO[•] from cells during oxidative stress. Alternatively (Kuo and Abe, 1996a; Luperchio et al., 1996), it has been suggested that NO[•] is involved in the regulation of GSH synthesis, upregulating GSH synthesis during times of oxidative stress. The data in chapter 4 shows that inducing iNOS and increasing NO[•] release is concomitant with a decrease in GSH, but this decrease is independent of NO[•] release, since inhibitors of NO[•] production do not prevent the decrease in intracellular GSH. It is therefore unlikely that NO[•] has any effect on the intracellular levels of GSH in our experimental system at 24 hours, the time point at which nitrite can be measured in the medium. Later time-points may, however, show decreases in GSH levels that are dependent on NO[•] release, since NO[•] can readily bind to thiol containing compounds such as GSH.

GSH may be involved in the induction of iNOS either directly, affecting activation of the transcription factors necessary for iNOS induction, or indirectly, by removing excessive ROS which may themselves affect the transcription factors for iNOS induction. It has been shown that activation of cells with TNF- α causes an increase in intracellular ROS, through its action on the mitochondria, (Duval et al., 1995), which release superoxide anions. Super oxide anions are, in turn, converted to H₂O₂ by SOD. The addition of H₂O₂ to the cells has a similar effect as TNF- α on the activation of iNOS. If ROS are involved in the induction of iNOS, then GSH could also be involved since it is a cofactor in the breakdown of H₂O₂ by GPx. This chapter has focused on the manipulation of GSH levels in both the A549 and 16HBE cells in order to determine the effect of GSH on iNOS induction, by both cytokines and H₂O₂.

Intracellular GSH can be decreased in cells by a variety of compounds. In this study buthionine sulfoximine (BSO) was used. BSO has been widely used to decrease GSH, especially in A549 cells, where it has been used to examine the importance of GSH in cell proliferation (see chapter 3)(Beasley et al., 1985; Ling et al., 1990). BSO has been used at various concentrations (50 μ M to 10mM) and the effects on intracellular GSH levels have been reported at time points ranging from 24 hours to 8 days (Kang, 1993; Kang, 1994; Ling et al., 1990; Poot et al., 1995). BSO has been shown to inhibit cell proliferation at 10mM (Kang, 1994). I therefore used a low concentration of 50 μ M BSO, in order to prevent inhibition of cell proliferation. My data shows that this concentration decreased intracellular GSH by approximately 90% of control levels after 16 hours, and that the decrease remained for at least 24 hours (GSH levels were not measured after 24 hours). My data also show that treatment of cells with BSO for 24 hours increased NO \cdot release from the cells to levels similar to cytomix. This does not agree with Hothersal *et al* (Hothersall et al., 1997), who reported that decreasing GSH with a variety of inhibitors, including BSO, also decreased NO \cdot output following activation with LPS and IFN- γ . In this report cells were pre-treated for 12 hours with BSO, at which point GSH levels were 30% of control cells, which resulted in a 40% decrease in NO \cdot production. Hothersal *et al* (Hothersall et al., 1997) hypothesised that this may be due to the L-arginine

transport system requiring thiols such as GSH for its function, since N-ethylmaleimide (NEM), a GSH-depleting compound can also inhibit L-arginine transport. In addition GSSG levels were thought to be important since GSSG can inhibit phosphorylation of proteins necessary for iNOS induction (Hothersall et al., 1997). I therefore studied the effect of pre-treating cells with BSO for 16 hours (at which point GSH levels were decreased by approximately 90%), followed by cytomix. In these experiments NO[•] levels were still increased in BSO-treated cells. Thus I did not observe an inhibition of NO[•] release by decreasing GSH levels. BSO is likely to induce NO[•] in my experimental system by depleting GSH and so preventing it reacting with and removing excess ROS. Organelles within the cell such as the mitochondria release ROS in aerobic conditions, and these are normally quenched by directly reacting with GSH. Reducing intracellular GSH levels may therefore result in an increase in the level of ROS above normal, which may switch on iNOS induction by an oxidant-mediated pathway.

Thus GSH appears to have a complex role in NO[•] induction. GSH may be necessary for induction of NO[•], and decreasing GSH levels causes inhibition of induction (Hothersall et al., 1997). Alternately it has been suggested that inhibition of NO[•], causes a decrease in GSH levels, and NO[•] may possibly have a role in the synthesis of GSH at a transcriptional level (Baeuerle et al., 1996). My data suggest that GSH may be involved in NO[•] induction either directly or indirectly in response to certain stimuli. NO[•] is induced by cytokines, one of which, TNF- α , has been shown to cause an increase in intracellular ROS (Duval et al., 1995). H₂O₂, which produces oxidant stress, and BSO, which decreases intracellular GSH, are also likely to result in increased intracellular ROS. It is therefore likely that NO[•] can be induced by an oxidant stress and that GSH may influence that induction indirectly by reducing ROS. The level of GSSG, in particular the ratio of GSSG:GSH, may also be important, by inhibiting the phosphorylation of proteins necessary for the induction of transcription factors involved in iNOS induction (Droge et al., 1994). The importance of the ratio of GSSG:GSH is discussed in more detail in Chapter 6.

In view of the potential deleterious affects of high amounts of NO[•] production by iNOS during inflammation (Anggard, 1994), the prevention of iNOS expression could be important therapeutically. Since evidence suggests that GSH is involved in iNOS induction I examined the effect of increasing intracellular GSH levels in the A549 and 16HBE lung epithelial cells.

Intracellular GSH was increased using the thiol compounds NAC, NAL, GSH, and GSHMEE, all of which are thiol containing compounds with antioxidant properties. Antioxidants are of interest in the treatment of diseases, particularly chronic inflammatory respiratory disease due to their ability to react with ROS. Both NAC and NAL have been shown to be potent scavengers of superoxide anions, H₂O₂, hydroxyl radicals and hypochlorous acid, (Arouma et al., 1989; Vanderbist et al., 1996), being equivalent in their scavenging abilities to GSH (Gillissen et al., 1997). GSHMEE is taken up by cells and its ester group is cleaved, on entering the cell, to release GSH. Increases in GSH levels produced by thiol compounds have previously been reported using such compounds and these increases have occurred at between 2 and 6 hours for NAC (Phelps et al., 1992), 3 hours for GSH (Chen et al., 1992), and 2-3 hours for GSHMEE (Anderson et al., 1985). My results show that all 4 thiol compounds significantly increased intracellular GSH levels at 4 hours to concentrations similar to these previously reported. A variety of antioxidants have previously been used (Hecker et al., 1996b) to inhibit cytokine induction of iNOS in vascular smooth muscle cells. However, although the antioxidants were capable of inhibiting superoxide anion production (by PMA), suggesting they were effective antioxidants, they did not necessarily inhibit IL-1 β induction of iNOS. This suggests that not all antioxidants are able to inhibit iNOS induction and that different antioxidants may act on different parts of the pathway involved in iNOS induction. In addition, stimulation of RAW 264.7 macrophages with LPS and IFN- γ has a synergistic effect on iNOS induction, and this effect can be inhibited by antioxidants that prevent activation of transcription factors (Hecker et al., 1996a). Thus it is likely that iNOS induction by certain cytokines contains a redox-sensitive step which can be inhibited by antioxidants. Production of oxidants is not always necessary for the induction of iNOS, since PMA (which induces superoxide anion production) had no

effect on NO[•] levels (Hecker et al., 1996a), suggesting that although there may be a redox sensitive step in the induction of iNOS, this may not be sufficient for NO[•] release.

Cells were therefore pre-treated with the thiol compounds, followed by cytomix or H₂O₂ to determine if increased GSH levels could inhibit iNOS induction. Cells pre-treated with 4 thiol compounds for 4 hours followed by cytomix for 24 hours released NO[•] at levels similar to those observed with cytomix alone. This suggests that either cytomix does not induce NO[•] by an oxidant mediated pathway or that GSH, at the levels produced by these thiol compounds does not diminish ROS activity sufficiently to interfere with ROS mediated NO[•] release. Pre-treating the cells with the 4 thiol compounds followed by H₂O₂ did, however, decrease NO[•] release compared to those observed with H₂O₂ alone. This suggests that NO[•] can be induced by an oxidant mediated pathway, and that this pathway can be inhibited by antioxidants which increase intracellular GSH levels. It is possible from these results that NO[•] can be induced in 2 ways, one by a direct effect of oxidants and one by an effect of cytokines which may bypass the oxidant step. Although TNF- α induces ROS release from the mitochondria, it may also act on another pathway synergistically with the other cytokines.

There are very few reports on the effect of GSH, or other antioxidants, and oxidants on NO[•] induction that do not focus on NF- κ B activation. The effect of antioxidants on NF- κ B activation and hence NO[•] induction are discussed in more detail in Chapter 6. The ratio of GSSG:GSH is also determined, in order to verify if it is important in iNOS induction.

Chapter 6 The effect of cytokines, H₂O₂ and modulating GSH levels on NF- κ B activation.

6.1 INTRODUCTION

The transcription factor NF- κ B is present in the cytosol of cells in an inactivated form, due to its association with the inhibitory subunit I κ B. Activation of NF- κ B requires the phosphorylation and ubiquitination of I κ B, which then dissociates from NF- κ B and is degraded. NF- κ B can then move to the nucleus, where it binds to DNA switching on gene transcription for a large number of molecules including iNOS and pro-inflammatory cytokines (Baeuerle and Baichwal, 1997; Baldwin, 1996; Rahman and MacNee, 1998) (see **Figure 7 of introduction**). NO synthesis has been shown to require NF- κ B activation for induction of iNOS (Ignarro and Murad, 1995; Wong et al., 1996; Droge et al., 1994), and although other transcription factors such as IRF-1 are thought to be required for optimal induction (Ignarro and Murad, 1995; Salkowski et al., 1996), NF- κ B is a key player in the induction of iNOS (Amoah-Apraku et al., 1995; Flodstrom et al., 1996). I therefore examined the effect of cytokines and H₂O₂ on NF- κ B activation in the cell. Increased ROS are thought to be involved in the activation of NF- κ B, since NF- κ B activation can be inhibited by antioxidants (Flohe et al., 1997; Sen and Packer, 1996). GSH may be involved in NF- κ B activation and in particular the ratio of GSSG:GSH may be critical (Droge et al., 1994; Galter et al., 1997). I therefore examined the effect of antioxidants which increase intracellular GSH on NF- κ B activation by oxidants (H₂O₂) and cytokines. The effect of a mitochondrial inhibitor, rotenone was also examined, to determine if blocking mitochondrial release of superoxide anions had any effect on oxidant and cytokine induced NF- κ B activation.

6.2 A TYPICAL GEL SHIFT

All the results of NF- κ B activation are captured images of original autoradiographs, allowing density of the bands to be determined by densitometry. Densitometry provides an idea of the level of activation above control levels, but huge variations occurred from gel to gel, and hence standard errors are often large and statistical analysis is therefore not carried out. In some figures below, bands have been cut out of the gel in order to be placed next to appropriate bands for that figure. In all cases, the bands are from the same gel, but gels contain up to 15 samples, many of which are irrelevant to that particular figure. Although differences may appear in some figures, this is due to the film background, which is not always uniform. Some autoradiographs were computer enhanced due to backgrounds being dark and images being unclear. Enhancement, however, only involved changing of background brightness and contrast in order to make the images clearer and was carried out on the whole autoradiograph before gels were cut. Figure 65 shows an example of a 'typical' gel shift autoradiograph.

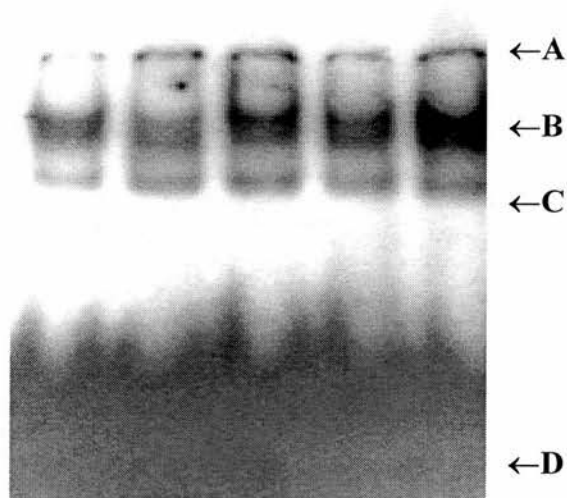


Figure 65: An example of a typical gel shift. **A** corresponds to the wells into which the samples are loaded, **B** corresponds to NF- κ B double shift bands, **C** corresponds to a non-specific band (see later), **D** corresponds to excess unbound radiolabeled NF- κ B oligonucleotide.

Since several bands were present on the gel shift, several methods were used to try and distinguish which bands were NF- κ B. Firstly rabbit polyclonal antibodies directed to the nuclear location sequence on the p50 and p65 subunits of NF- κ B were used. The antibody was added to samples treated with cytomix before the addition of the labeled oligonucleotide. The antibody binds to the relevant sub-unit, and due to the net increase in the overall molecular weight, 'super-shifts' to a different part of the gel are observed. Figure 66 shows the effect of addition of either the p50 or p65 antibody to A549 cells treated with cytomix. The intensity of the band that is increased in cytomix-treated cells is decreased in both p65 and p50 antibody treated reactions, more so in the p65 antibody treated reactions, and the band appears to have 'supershifted'. This suggests that the band thought to be NF- κ B contains both the p65 and p50 subunits of NF- κ B. Two bands are present in this gel, the p65 seems to decrease the top band, and the p50 the bottom band, suggesting that the protein may degrade slightly to give two bands. There are also several small bands within the main bands, and these may also be due to degradation of the protein.

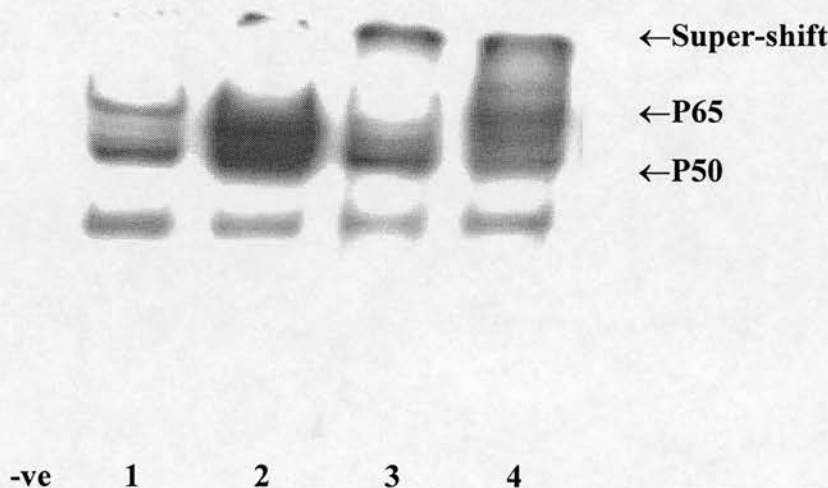


Figure 66: the effect of using antibodies against the p50 and p65 subunits of NF- κ B in cytomix treated A549 cells. **-ve control**; **Lane 1**: control; **Lane 2**: cytomix; **Lane 3**: cytomix with the p65 antibody; **Lane 4**: cytomix with the p50 antibody.

In order to clarify that these two bands (which often appear as one large band) represented the activated NF- κ B, recombinant I κ B α was used. I κ B α will bind to the p65 subunit of NF- κ B, and therefore inhibit binding of NF- κ B to the

oligonucleotide by masking the DNA binding sequence. Excess I κ B α was added to the samples prior to addition of the radiolabeled oligonucleotide. Figure 67 shows the effect of the addition of the I κ B α protein to both control and cytomix-treated A549 cell samples. The intensity of the band induced by cytomix is reduced to that of control cells without the protein. Control cells with the I κ B α protein show no difference to control cells without. This suggests that the increase in the intensity of the band observed with cytomix contains a p65 subunit, and could be either a p65/p50 NF- κ B or a p65/p65 homodimer. The band remaining could be a p50 subunit, or a p50/p50 homodimer, since I κ B α does not bind to the p50 subunit.

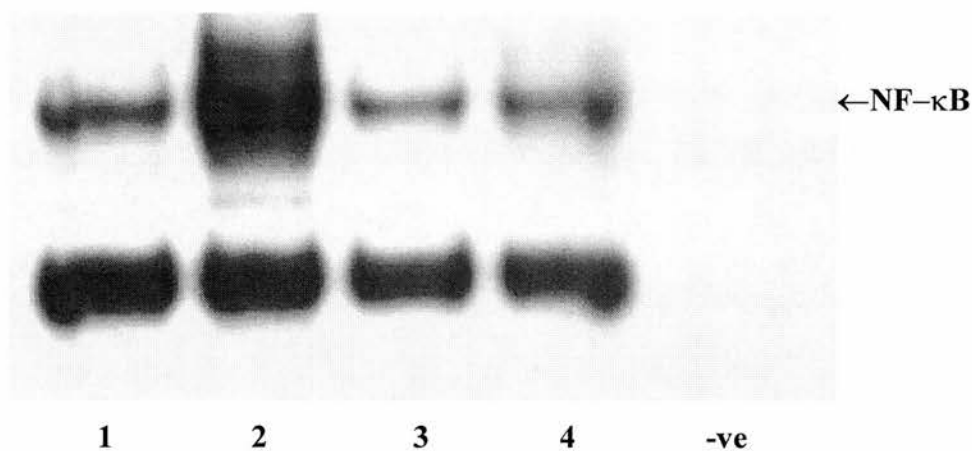


Figure 67: The effect of adding excess, unlabeled I κ B α recombinant protein to samples prior to addition of labeled NF- κ B oligonucleotide. **Lane 1:** control; **Lane 2:** cytomix; **Lane 3:** control with I κ B α ; **Lane 4:** cytomix with I κ B α , **-ve control**.

The bottom band that appears in the gels is not altered in any of the gels following addition of the antibodies or recombinant I κ B α protein, suggesting that it is not a NF- κ B protein, or is a product of denaturation. In a cold displacement assay (which involves incubation with excess unlabelled oligonucleotide) this band has been shown to be a product of non-specific binding (Mark Lawson, Rayne Laboratory).

Densitometry was therefore measured on the top 2 bands, which are often indistinguishable from one another.

6.3 THE EFFECT OF CYTOMIX, INDIVIDUAL CYTOKINES AND LPS ON NF- κ B ACTIVATION.

Both cells lines were treated for 30 minutes with cytomix, TNF- α , IL1- β and IFN- γ (all at 10ng/ml). Figures 68 and 69 show the effect of treating both A549 and 16HBE cells with cytomix and individual cytokines. Cytomix, TNF- α and IL1- β all increased NF- κ B activation above control levels in both A549 and 16HBE cells. IFN- γ did not have any obvious effect on NF- κ B activation by itself. Figure 70 shows the relative densitometry of NF- κ B activation in cytokine treated cells.

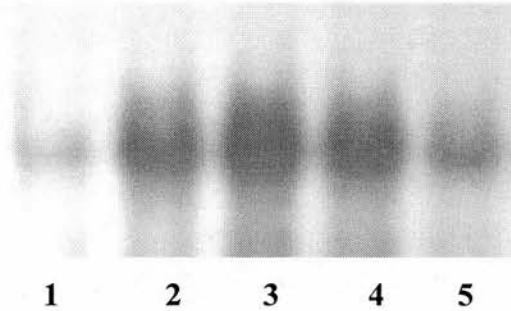


Figure 68 : The effect of cytomix and individual cytokines on NF- κ B activation in A549 cells. **Lane 1:** control; **Lane 2:** cytomix; **Lane 3:** TNF- α 10ng/ml; **Lane 4:** IL1- β 10ng/ml; **Lane 5:** IFN- γ 10ng/ml.

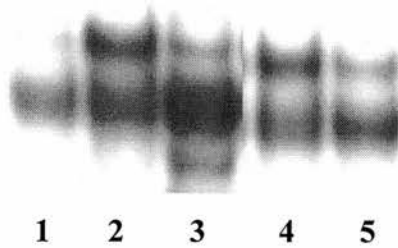


Figure 69: The effect of cytomix and individual cytokines on NF- κ B activation in 16HBE cells. **Lane 1:** control; **Lane 2:** cytomix; **Lane 3:** TNF- α 10ng/ml; **Lane 4:** IL1- β 10ng/ml; **Lane 5:** IFN- γ 10ng/ml.

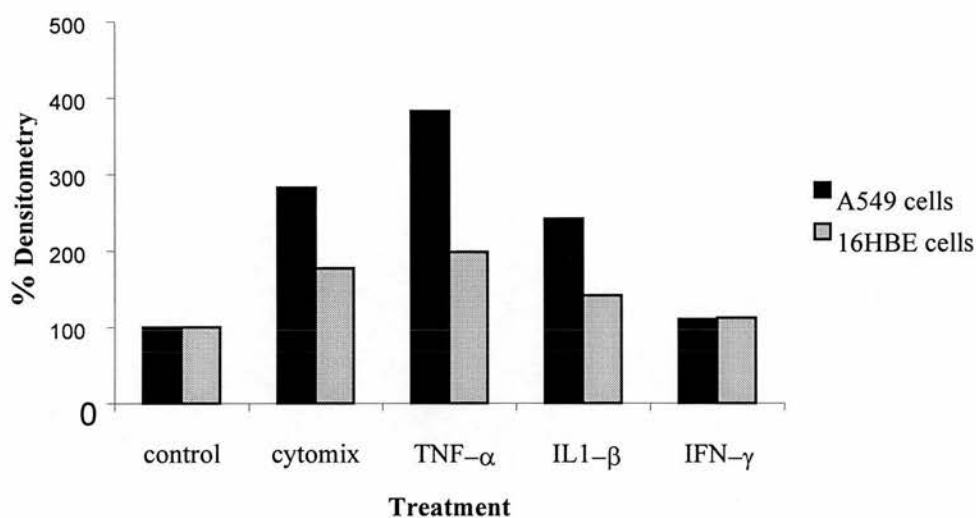


Figure 70: The effect of treating A549 and 16HBE cells with cytomix and the individual cytokines on NF- κ B activation as measured by densitometry.

Both cell lines were also treated with LPS at 1 μ g/ml for 30 minutes. Figure 71 shows the effect of LPS on NF- κ B activation in both A549 and 16HBE cells. LPS had no effect on NF- κ B activation in A549 cells but increased NF- κ B activation in 16HBE cells. Figure 72 shows the relative densitometry of NF- κ B activation in LPS treated cells.

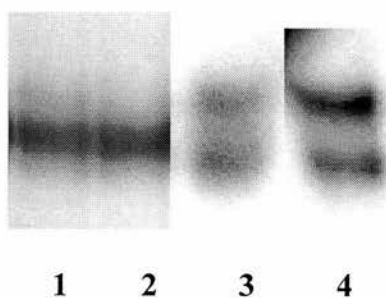


Figure 71: The effect of LPS on NF- κ B activation in both A549 and 16HBE cells at 30 minutes. **Lane 1:** control A549 cells; **Lane 2:** LPS A549 cells, **Lane 3:** control 16HBE cells; **Lane 4:** LPS 16HBE cells.

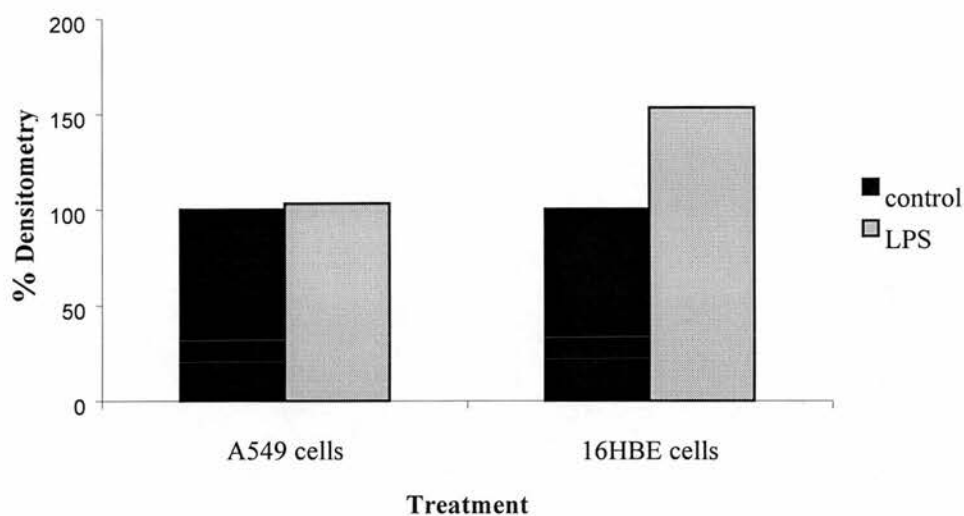


Figure 72: The effect of treating A549 and 16HBE cells with LPS on NF-κB activation as measured by densitometry.

Both A549 and 16HBE cell lines were treated with 1μM H₂O₂ (the concentration used to increase nitrite production significantly in both cell types) to investigate the effect of oxidants on NF-κB activation. A 2 hour time point was chosen since in our laboratory it has previously been shown that this time-point is the earliest time point at which there is an increase in NF-κB activation following this concentration of H₂O₂. Figure 73 shows the relative densitometry of NF-κB activation by H₂O₂ at 30 minutes and 2 hours as measured by Dr. B. Mulier.

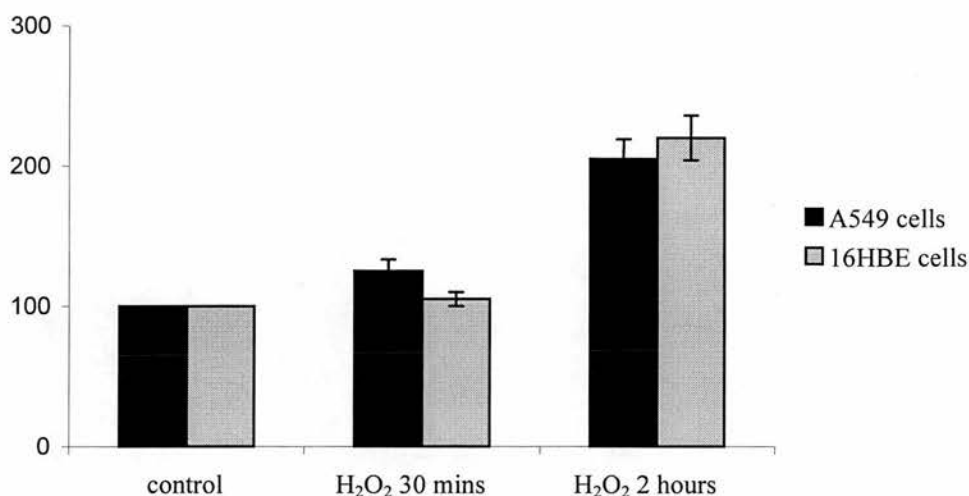


Figure 73: The effect of treating A549 and 16HBE cells with H₂O₂ on NF-κB activation at 30 minutes and 2 hours, as measured by densitometry (n=4).

Both A549 and 16HBE cells were thus treated with H₂O₂ for 2 hours to determine the effect on NF-κB activation (figure 74). NF-κB nuclear binding is increased in both A549 and 16HBE cells by H₂O₂ at 2 hours. Figure 75 shows the relative densitometry of NF-κB activation in H₂O₂ treated cells.

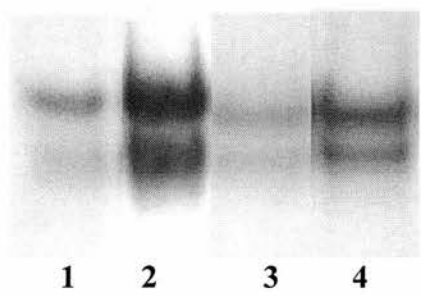


Figure 74: The effect of H₂O₂ on NF-κB activation in both A549 and 16HBE cells at 2 hours. **Lane 1:** control A549 cells; **Lane 2:** H₂O₂ A549 cells; **Lane 3:** control 16HBE cells; **Lane 4:** H₂O₂ 16HBE cells.

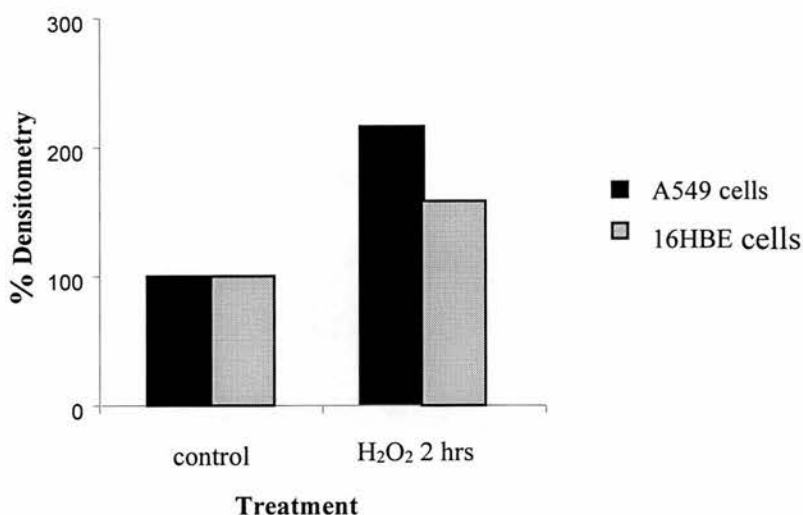


Figure 75: The effect of treating A549 and 16HBE cells with H₂O₂ on NF-κB activation as measured by densitometry.

6.4 THE EFFECT OF MODULATING INTRACELLULAR GSH LEVELS BY BSO AND THIOL COMPOUNDS ON NF-κB ACTIVATION.

Since the compound BSO resulted in both an increase in nitrite production and an induction of iNOS mRNA, I examined the effects of BSO on NF-κB activation in both A549 and 16HBE cells. NF-κB activation was investigated at different time points, from 30 minutes to 4 hours. Figures 76 and 77 show the effect of BSO at different time points on NF-κB activation in both A549 and 16HBE cells. BSO activates NF-κB in A549 cells at 4 hours in 16HBE cells at 2 and 4 hours. Figure 78 shows the relative densitometry of NF-κB activation in BSO treated cells.

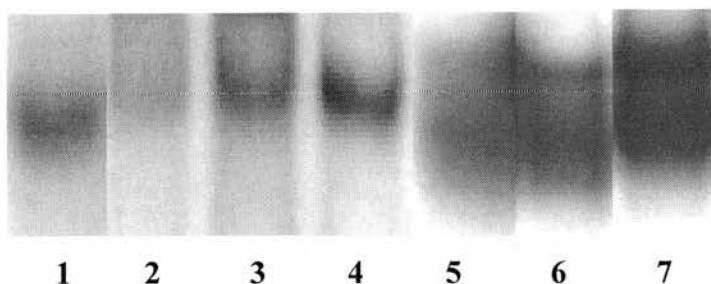


Figure 76 : The effect of BSO on NF- κ B activation in A549 cells. **Lane 1:** control; **Lane 2:** BSO 30 minutes; **Lane 3:** BSO 1 hour; **Lane 4:** BSO 4 hours; **Lane 5:** control; **Lane 6:** BSO 2 hours; **Lane 7:** BSO 4 hours.

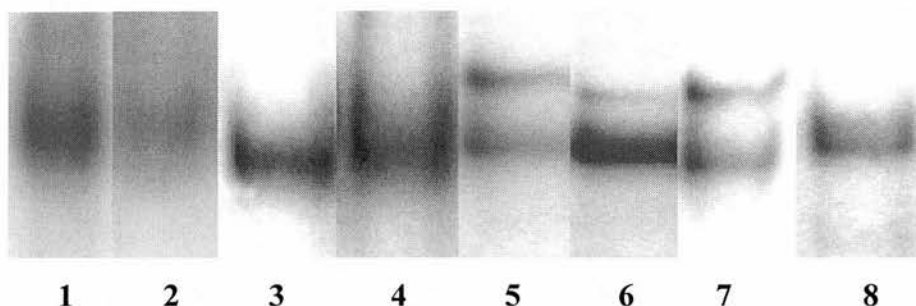


Figure 77: The effect of BSO on NF- κ B activation in 16HBE cells. **Lane 1:** control; **Lane 2:** BSO 30 minutes; **Lane 3:** control; **Lane 4:** BSO 1 hours; **Lane 5:** control; **Lane 6:** BSO 2 hours, **Lane 7:** control, **Lane 8:** BSO 4 hours.

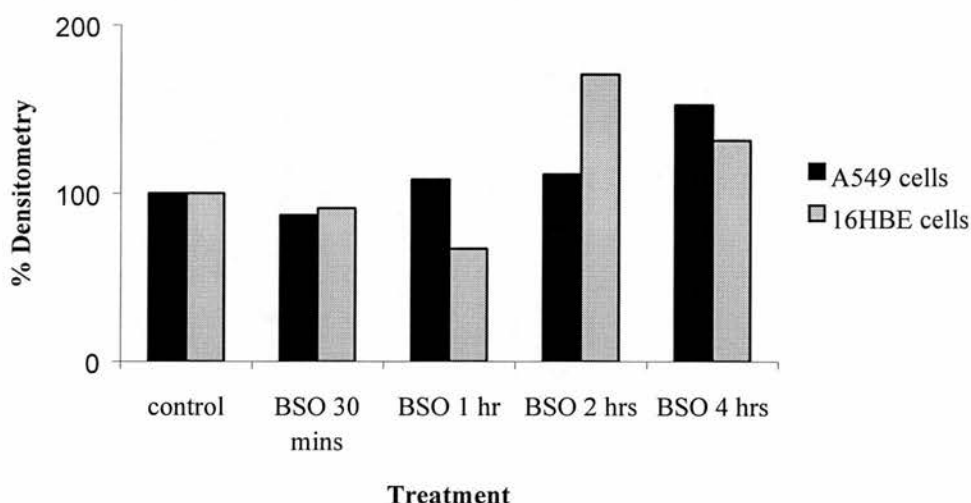


Figure 78: the effect of treating A549 and 16HBE cells with BSO at various time points on NF- κ B activation as measured by densitometry.

The 4 thiol compounds used previously to increase intracellular GSH levels were also used in order to study the effect of increasing GSH on cytokine and H₂O₂ induced NF- κ B activation. Figures 79 and 80 show the effect of treating both cell lines with NAC, NAL, GSH and GSHMEE, on NF- κ B activation at the 4 hour time point, the time at which intracellular GSH levels are increased. The 4 thiol compounds have no effect on NF- κ B activation alone. Figure 81 shows the relative densitometry of NF- κ B treated with the 4 thiol compounds.

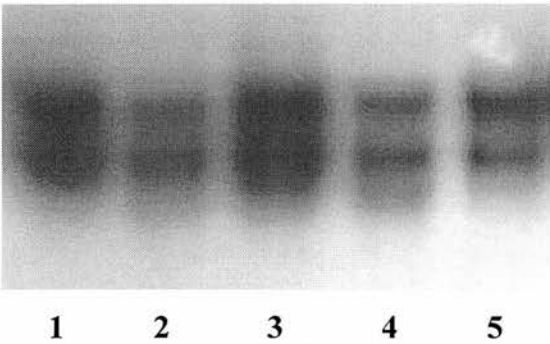


Figure 79: The effect of the 4 thiol compounds, NAC, NAL, GSH, and GSHMEE on NF- κ B activation in A549 cells. **Lane 1:** control; **Lane 2:** NAC; **Lane 3:** NAL; **Lane 4:** GSH; **Lane 5:** GSHMEE.

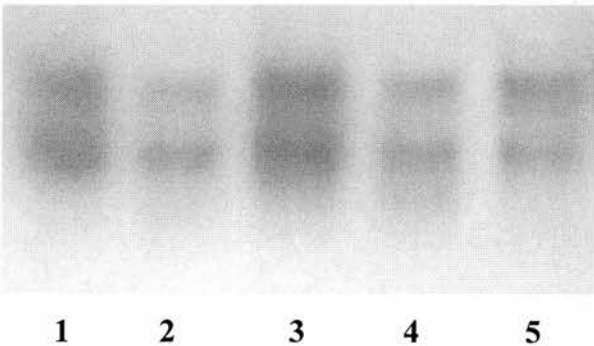


Figure 80: The effect of the 4 thiol compounds, NAC, NAL, GSH, and GSHMEE on NF- κ B activation in 16HBE cells. **Lane 1:** control; **Lane 2:** NAC; **Lane 3:** NAL; **Lane 4:** GSH; **Lane 5:** GSHMEE.

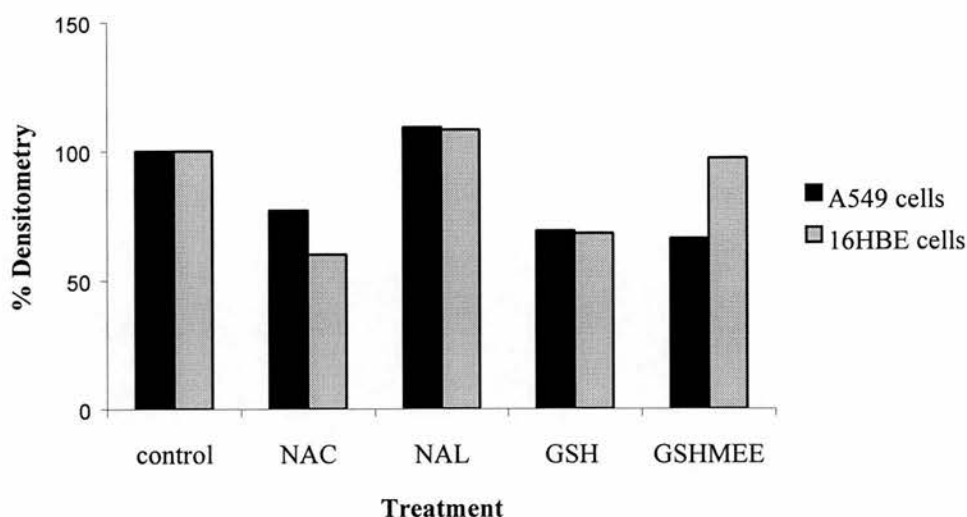


Figure 81: The effect of treating A549 and 16HBE cells with the 4 thiol compounds on NF- κ B activation as measured by densitometry.

The cells were then pre-treated with the 4 thiol compounds for 4 hours followed by cytomix or TNF- α for 30 minutes or by H₂O₂ for 2 hours to determine the effect of increased GSH levels on cytokine and oxidant induced NF- κ B activation. Figures 82 and 83 show the effect of pre-treating cells with thiol compounds for 4 hours followed by cytomix for 30 minutes on NF- κ B activation in both A549 and 16HBE cells. Cytomix increases NF- κ B activation at 30 minutes. There is no difference between cytomix alone and cells pre-treated with thiol compounds followed by cytomix, indicating that the increase in GSH levels produced by these compounds do not prevent cytomix induced NF- κ B activation. Figure 84 shows the relative densitometry of NF- κ B activation in cells pre-treated with thiol compounds followed by cytomix.

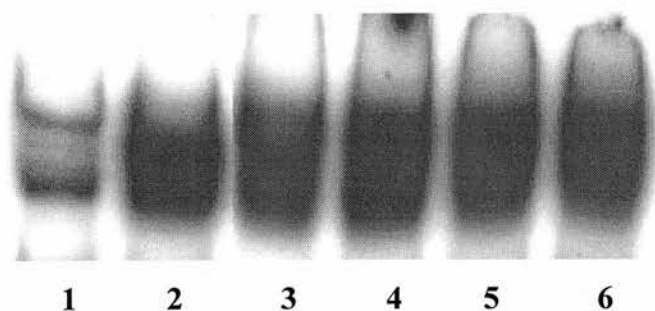


Figure 82: The effect of pre-treating A549 cells for 4 hours with the 4 thiol compounds followed by cytomix for 30 minutes on NF- κ B activation. **Lane 1:** control; **Lane 2:** cytomix; **Lane 3:** NAC + cytomix; **Lane 4:** NAL + cytomix; **Lane 5:** GSH + cytomix; **Lane 6:** GSHMEE + cytomix.

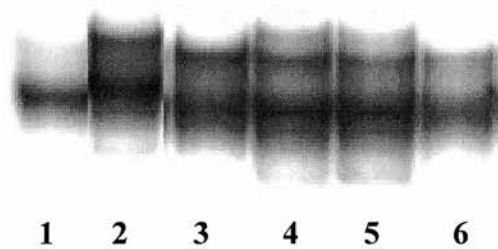


Figure 83: The effect of pre-treating 16HBE cells with the 4 thiol compounds followed by cytomix for 30 minutes on NF- κ B activation. **Lane 1:** control; **Lane 2:** cytomix; **Lane 3:** NAC + cytomix ; **Lane 4:** NAL + cytomix ; **Lane 5:** GSH + cytomix ; **Lane 6:** GSHMEE + cytomix .

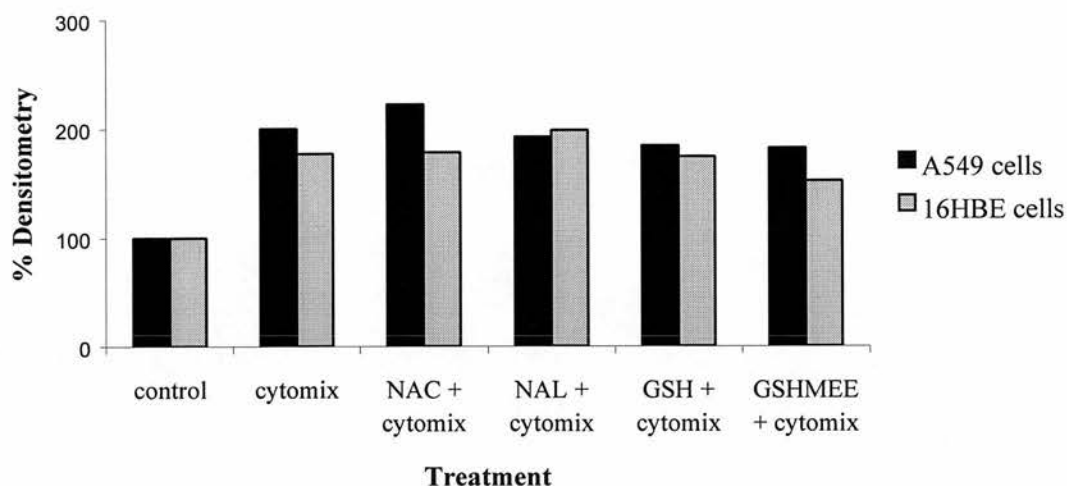


Figure 84: The effect of treating A549 and 16HBE cells with the 4 thiol compounds followed by cytomix on NF- κ B activation as measured by densitometry .

Cells were then pre-treated with the 4 thiol compounds followed by H_2O_2 . Figures 85 and 86 show the effect of pre-treating cells with 4 thiol compounds for 4 hours followed by H_2O_2 for 2 hours on NF- κ B activation in both A549 and 16HBE cells. In both cell types, H_2O_2 induced NF- κ B activation was decreased following pre-treatment with the thiol compounds. Figure 87 shows the relative densitometry of NF- κ B activation in cells pre-treated with thiol compounds followed by H_2O_2 .

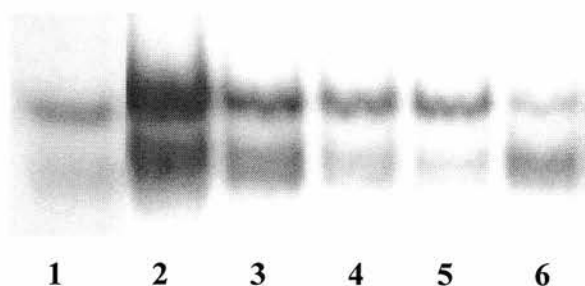


Figure 85: The effect of pre-treating A549 cells for 4 hours with the 4 thiol compounds followed by H_2O_2 for 2 hours on NF- κ B activation. **Lane 1:** control; **Lane 2:** H_2O_2 ; **Lane 3:** NAC + H_2O_2 ; **Lane 4:** NAL + H_2O_2 ; **Lane 5:** GSH + H_2O_2 ; **Lane 6:** GSHMEE + H_2O_2 .

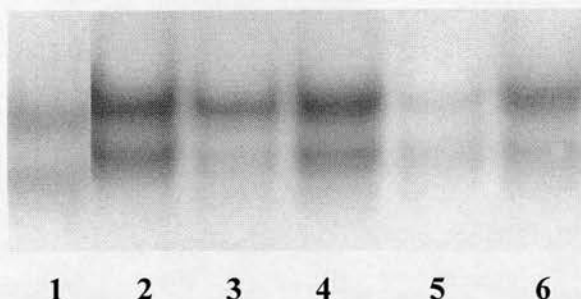


Figure 86: The effect of pre-treating 16HBE cells for 4 hours with the 4 thiol compounds followed by H_2O_2 for 2 hours on NF- κ B activation. **Lane 1:** control; **Lane 2:** H_2O_2 ; **Lane 3:** NAC + H_2O_2 ; **Lane 4:** NAL + H_2O_2 ; **Lane 5:** GSH + H_2O_2 ; **Lane 6:** GSHMEE + H_2O_2 .

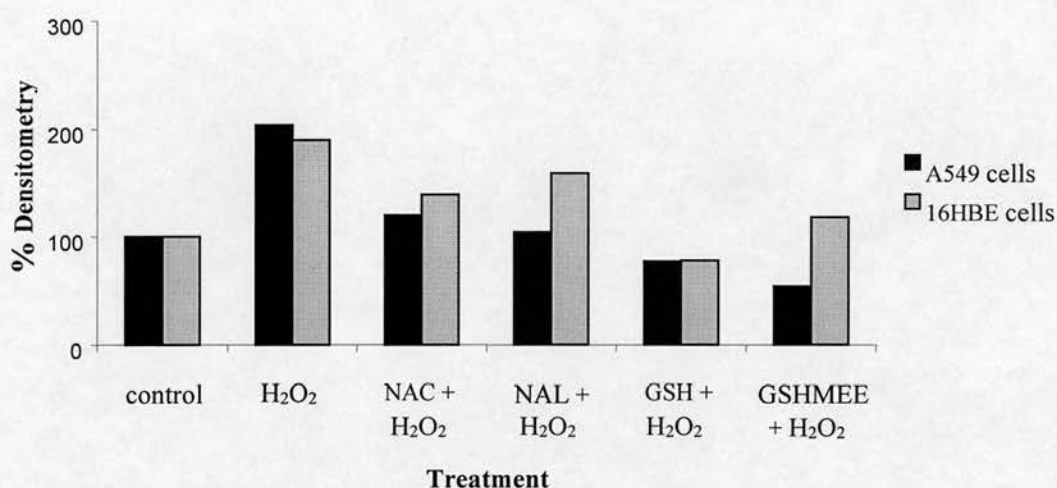


Figure 87: The effect of treating A549 and 16HBE cells with the 4 thiol compounds followed by H_2O_2 on NF- κ B activation as measured by densitometry .

Since increased GSH levels did not decrease cytokine induced NF- κ B activation I investigated the effect of pre-treating cells with the 4 thiol compounds followed by TNF- α . TNF- α has been shown to induce NF- κ B activation by increasing intracellular ROS levels due to its action on the mitochondria. Figures 88 and 89 show the effect of pre-treating cells with the 4 thiol compounds followed by TNF- α alone at 10ng/ml for 30 minutes on NF- κ B activation in both A549 and 16HBE cells. Increasing GSH levels appeared to decrease TNF- α induced NF- κ B in A549

cells pre-treated with NAL and GSH, and in 16HBE cell pre-treated with NAL. Other pre-treatments, however, did not decrease NF- κ B activation induced by TNF- α in either cell type. Figure 90 shows the relative densitometry of NF- κ B activation in cell pre-treated with thiol compounds followed by TNF- α .

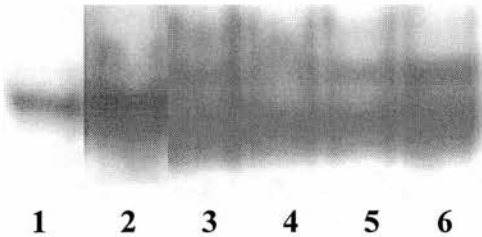


Figure 88: The effect of pre-treating A549 cells with the 4 thiol compounds followed by TNF- α for 30 minutes on NF- κ B activation. **Lane 1:** control; **Lane 2:** TNF- α ; **Lane 3:** NAC + TNF- α ; **Lane 4:** NAL + TNF- α ; **Lane 5:** GSH + TNF- α ; **Lane 6:** GSHMEE + TNF- α .

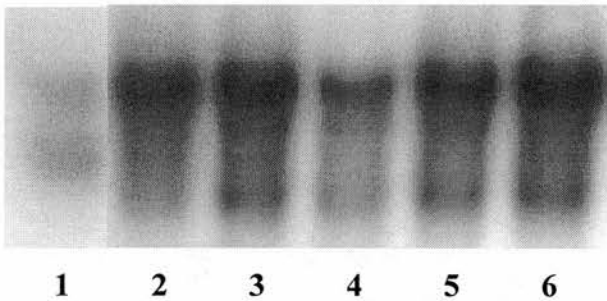


Figure 89: The effect of pre-treating 16HBE cells with the 4 thiol compounds followed by TNF- α for 30 minutes on NF- κ B activation. **Lane 1:** control; **Lane 2:** TNF- α ; **Lane 3:** NAC + TNF- α ; **Lane 4:** NAL + TNF- α ; **Lane 5:** GSH + TNF- α ; **Lane 6:** GSHMEE + TNF- α .

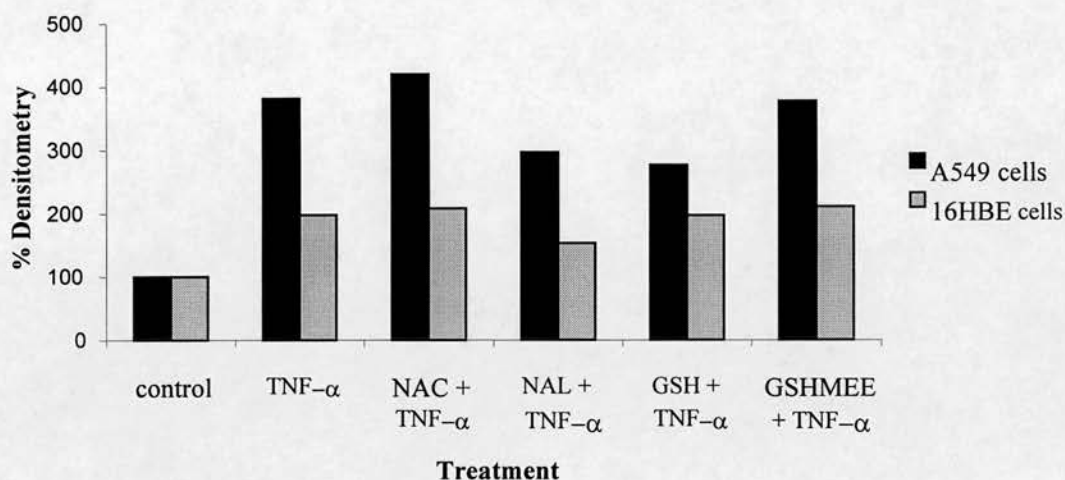


Figure 90: The effect of treating A549 and 16HBE cells with the 4 thiol compounds followed by TNF- α on NF- κ B activation as measured by densitometry .

6.5 THE EFFECT OF ROTENONE ON CYTOKINE AND H₂O₂ INDUCED NF- κ B ACTIVATION.

Rotenone is a mitochondrial inhibitor, inhibiting complex I of the electron transport system, and thus inhibits the flow of electrons that reduce oxygen to water. Rotenone can therefore inhibit the production of superoxide anions, since oxygen is not partially reduced, and hence inhibits H₂O₂ production, which is produced by superoxide anions and SOD. If NF- κ B activation is due to superoxide anion release from the mitochondria, then rotenone will inhibit this NF- κ B activation. Rotenone was dissolved in chloroform and the cells were pre-treated for 30 minutes followed by either cytomix or TNF- α for 30 minutes or H₂O₂ for 2 hours. The expression of iNOS mRNA was also assessed when the cells were pre-treated for 30 minutes with rotenone followed by cytomix TNF- α or H₂O₂ for 4 hours.

Figure 91 shows the effect on NF- κ B activation of pre-treating both A549 and 16HBE cells with rotenone followed by cytomix. Rotenone had no effect on cytomix

induced NF- κ B activation in either cell type. Figure 92 shows the relative densitometry of NF- κ B activation in cells pre-treated with rotenone followed by cytomix.

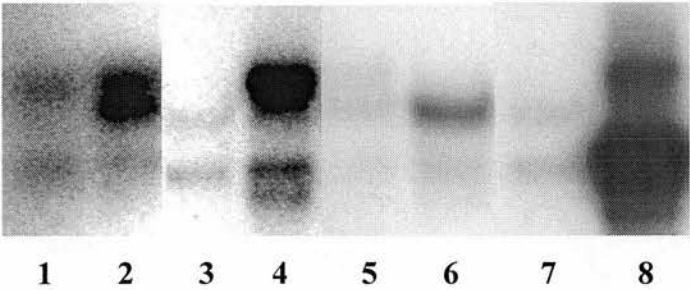


Figure 91: The effect of pre-treating both A549 and 16HBE cells with rotenone followed by cytomix for 30 minutes. **Lane 1:** control A549 cells; **Lane 2:** cytomix A549 cells; **Lane 3:** rotenone A549 cells; **Lane 4:** rotenone + cytomix A549 cells; **Lane 5:** control 16HBE cells; **Lane 6:** cytomix 16HBE cells; **Lane 7:** rotenone 16HBE cells; **Lane 8:** rotenone + cytomix 16HBE cells.

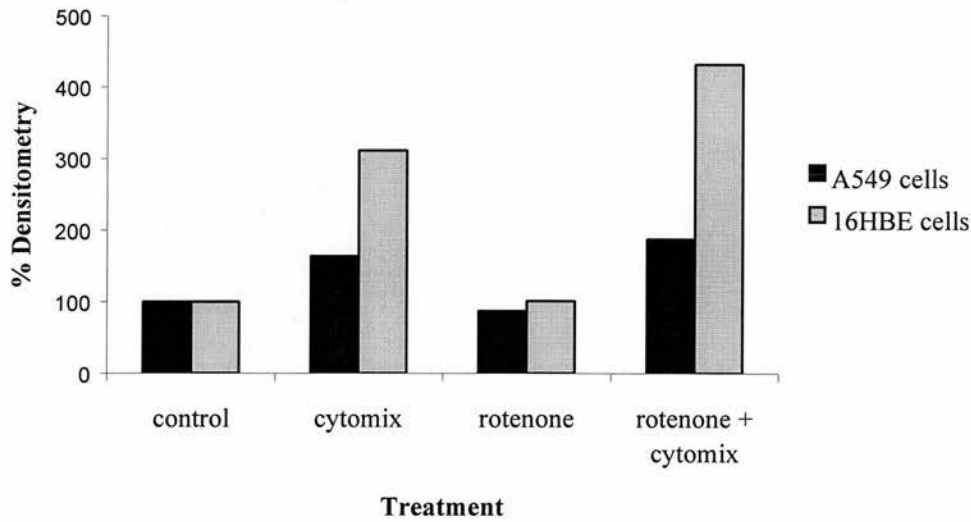


Figure 92: The effect of pre-treating both A549 and 16HBE cells with rotenone followed by cytomix for 30 minutes on NF- κ B activation as measured by densitometry.

Figure 93 shows the effect of pre-treating both A549 and 16HBE cells with rotenone followed by H₂O₂. Rotenone had no effect on H₂O₂ activation of NF-κB in either cell type. Figure 94 shows the relative densitometry of NF-κB activation in cells pre-treated with rotenone followed by H₂O₂.

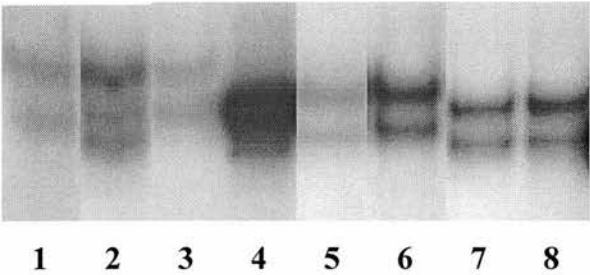


Figure 93: The effect of pre-treating both A549 and 16HBE cells with rotenone followed by H₂O₂ for 2 hours. **Lane 1:** control A549 cells; **Lane 2:** H₂O₂ A549 cells; **Lane 3:** rotenone A549 cells; **Lane 4:** rotenone + H₂O₂ A549 cells; **Lane 5:** control 16HBE cells; **Lane 6:** H₂O₂ 16HBE cells; **Lane 7:** rotenone 16HBE cells; **Lane 8:** rotenone + H₂O₂ 16HBE cells.

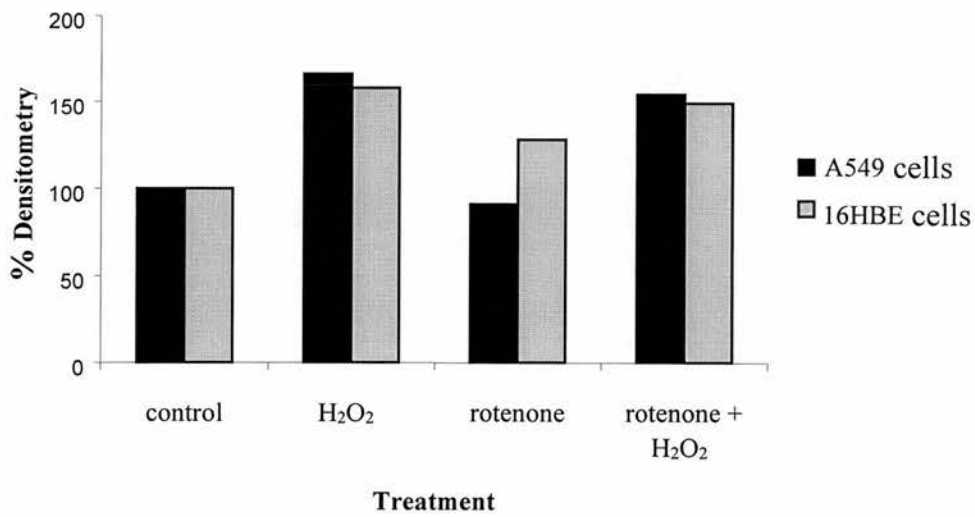


Figure 94: The effect of pre-treating both A549 and 16HBE cells with rotenone followed by H₂O₂ for 2 hours on NF-κB activation as measured by densitometry .

Figure 95 shows the effect of pre-treating both A549 and 16HBE cells with rotenone followed by TNF- α for 30 minutes on NF- κ B activation. Rotenone reduces TNF- α induced NF- κ B activation in both cell types. Figure 96 shows the relative densitometry of NF- κ B activation in cells pre-treated with rotenone followed by TNF- α .

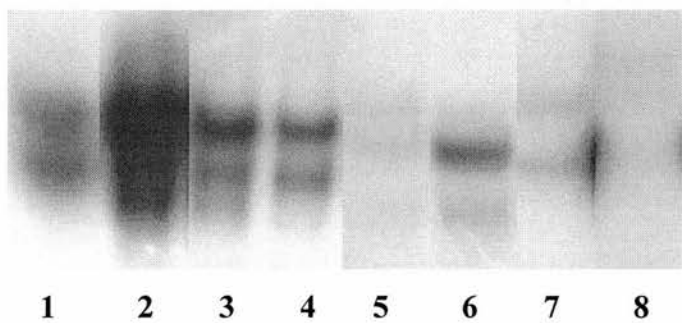


Figure 95: The effect of pre-treating both A549 and 16HBE cells with rotenone followed by TNF- α for 30 minutes. **Lane 1:** control A549 cells; **Lane 2:** TNF- α A549 cells; **Lane 3:** rotenone A549 cells; **Lane 4:** rotenone + TNF- α A549 cells; **Lane 5:** control 16HBE cells; **Lane 6:** TNF- α 16HBE cells; **Lane 7:** rotenone 16HBE cells; **Lane 8:** rotenone + TNF- α 16HBE cells.

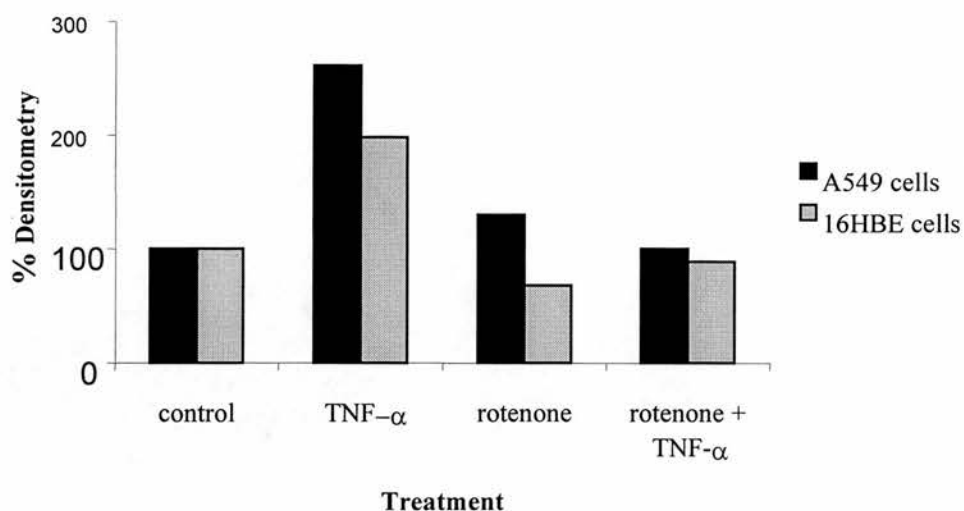


Figure 96: The effect of pre-treating both A549 and 16HBE cells with rotenone followed by TNF- α for 30 minutes on NF- κ B activation as measured by densitometry .

The above densitometry graphs are representative of the gel shown. Tables 2-5 show the densitometries of the above experiments for 3 separate experiments. Since the measurement is only semi-quantitative and values can vary hugely between different gels, statistical analysis is not carried out. However, the trend for each experiment is the same and the mean and SEM was measured for each experiment.

Table 2 The effect of cytokines, LPS and H₂O₂ on NF-κB activation in both A549 and 16HBE cells.

Treatment (30 minutes)	A549 cells	16HBE cells
Control	100	100
Cytomix	282, 312, 291	177, 161, 351
Average ± SEM	295 ± 9	230 ± 60
TNF-α	381, 219, 261	198, 165, 200
Average ± SEM	287 ± 48	187 ± 11
IL1-β	241, 315, 257	141, 163, 135
Average ± SEM	271 ± 22	146 ±9
IFN-γ	74, 110, 135	126, 61, 112
Average ± SEM	106 ± 18	99 ± 20
LPS	94, 103, 91	153, 140, 124
Average ± SEM	96 ± 4	139 ± 8
H ₂ O ₂ (30 minutes)	115, 74, 131,	77, 135, 113
Average ± SEM	106 ± 17	108 ± 16
H ₂ O ₂ (2 hours)	214, 256, 150	142, 146, 158
Average ± SEM	206 ± 31	149 ± 5

Table 3 The effect of BSO on NF-κB activation in both A549 and 16HBE cells at different time points.

Treatment	A549 cells	16HBE cells
Control	100	100
BSO 30 minutes	87, 108, 126	91, 101, 77
Average ± SEM	107 ± 11	90 ± 7
BSO 1 hour	108, 117, 87	38, 67, 54
Average ± SEM	104 ± 9	53 ± 8
BSO 2 hours	51, 111, 126	451, 170, 129
Average ± SEM	96 ± 23	250 ± 101
BSO 4 hours	133, 132, 152	131, 325, 114
Average ± SEM	139 ± 7	190 ± 67

Table 4 The effect of the 4 thiol compounds on cytomix, TNF-α and H₂O₂ induced NF-κB activation in both A549 and 16HBE cells

Treatment	A549 cells	16HBE cells
Control	100	100
NAC	73, 72, 77,	105, 60, 123
Average ± SEM	74 ± 1.5	96 ± 18
NAL	72, 109, 100	107, 108, 125
Average ± SEM	93 ± 11	113 ± 6
GSH	48, 133, 69	97, 68, 107
Average ± SEM	83 ± 26	90 ± 11
GSHMEE	45, 55, 66	47, 74, 97
Average ± SEM	55 ± 6	72 ± 14
Cytomix	291, 312, 282	177, 396, 1900
Average ± SEM	295 ± 9	824 ± 541
NAC + cytomix	228, 372, 222	178, 179, 2370
Average ± SEM	274 ± 49	909 ± 730

Treatment	A549 cells	16HBE cells
NAL + cytomix	192, 122, 424	198, 218, 2370
Average \pm SEM	246 \pm 72	928 \pm 720
GSH + cytomix	184, 272, 282	174, 204, 984
Average \pm SEM	279 \pm 31	424 \pm 265
GSHMEE + cytomix	182, 343, 4 \pm 7435	152, 149, 2000
Average \pm SEM	320 \pm 74	767 \pm 616
H ₂ O ₂	204, 166, 214	190, 384, 158
Average \pm SEM	194 \pm 14	244 \pm 70
NAC + H ₂ O ₂	120, 101, 66	85, 139, 77
Average \pm SEM	96 \pm 15	133 \pm 27
NAL + H ₂ O ₂	51, 104, 97	126, 159, 97
Average \pm SEM	84 \pm 16	127 \pm 17
GSH + H ₂ O ₂	31, 77, 46	78, 127, 116
Average \pm SEM	52 \pm 13	107 \pm 14
GSHMEE + H ₂ O ₂	54 ,142, 49	75, 136, 118
Average \pm SEM	82 \pm 30	109 \pm 18
TNF- α	381, 140, 144	198. 167, 1504
Average \pm SEM	221 \pm 80	623 \pm 440
NAC + TNF- α	419, 134, 137	208, 148, 1900
Average \pm SEM	230 \pm 94	725 \pm 574
NAL + TNF- α	296, 122, 133	153, 127, 1038
Average \pm SEM	183 \pm 56	439 \pm 299
GSH + TNF- α	276, 125, 125	197, 153, 813
Average \pm SEM	175 \pm 50	387 \pm 213
GSHMEE + TNF- α	376, 136, 124	211, 149, 1305
Average \pm SEM	212 \pm 82	555 \pm 375

Table 5 The effect of rotenone on cytomix, TNF- α and H₂O₂ induced NF- κ B activation in both A549 and 16HBE cells.

Treatment	A549 cells	16HBE cells
Control	100	100
Cytomix	164, 129, 126	351, 385, 481
Average \pm SEM	139 \pm 12	405 \pm 39
TNF- α	261, 153, 470	203, 198, 165
Average \pm SEM	295 \pm 93	188 \pm 12
H ₂ O ₂	150, 166, 166	158, 384, 146
Average \pm SEM	160 \pm 5	230 \pm 77
Rotenone	91, 87, 130	128, 68, 101
Average \pm SEM	102 \pm 14	99 \pm 17
Rotenone + cytomix	187, 124, 125	431, 801, 875
Average \pm SEM	144 \pm 23	702 \pm 137
Rotenone + TNF- α	65, 58, 100	42, 149, 89
Average	74 \pm 13	93 \pm 31
Rotenone + H ₂ O ₂	154, 132, 197	123, 446, 149
Average \pm SEM	161 \pm 19	239 \pm 103

6.6 THE EFFECT OF ROTENONE ON iNOS mRNA LEVELS.

Both A549 and 16HBE cells were pre-treated with rotenone for 30 minutes, followed by cytomix, TNF- α and H₂O₂ for 4 hours and iNOS mRNA levels measured. Unfortunately cell death was quite high in the 16HBE cells, (many cells were floating in the medium) possibly due to the effect of rotenone and chloroform for 4 1/2 hours, and therefore no results were obtained for rotenone and iNOS in 16HBE cells. The effects of rotenone on iNOS induction was measured, however, in A549 cells and figure 97 shows the effect of pre-treating cells with rotenone followed by cytomix, TNF- α and H₂O₂ for 4 hours. Cytomix, TNF- α , and H₂O₂ all induce iNOS mRNA, rotenone by itself has no effect; rotenone with cytomix and H₂O₂ has no

effect on cytomix and H₂O₂ induced iNOS; but rotenone and TNF- α prevents TNF- α induced iNOS supporting the evidence observed with NF- κ B activation. Figure 98 shows the ratio of iNOS to GAPDH.

iNOS



GAPDH

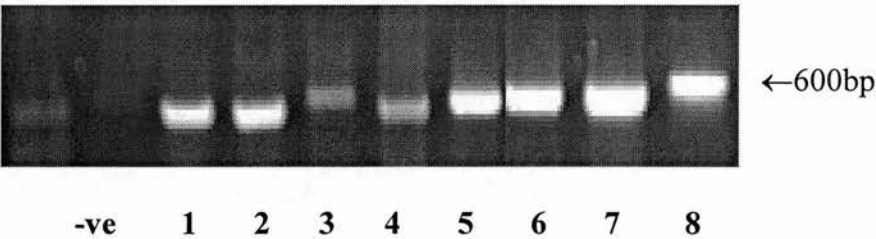


Figure 97: The effect of pre-treating A549 cells with rotenone followed by cytomix, TNF- α and H₂O₂ on iNOS and GAPDH mRNA levels.-ve control; +ve control; **Lane 1:** control; **Lane 2:** cytomix; **Lane 3:** TNF- α ; **Lane 4:** H₂O₂; **Lane 5:** rotenone; **Lane 6:** rotenone + cytomix; **Lane 7:** rotenone + TNF- α ; **Lane 8:** rotenone + H₂O₂.

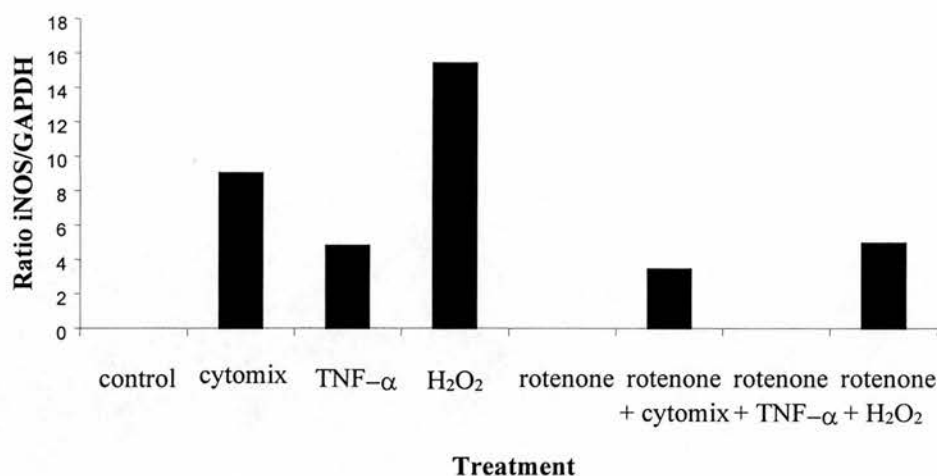


Figure 98: The ratio of iNOS to GAPDH in A549 cells pre-treated with rotenone followed by cytomix, TNF- α and H₂O₂. (n=1).

6.7 INTRACELLULAR GSSG:GSH RATIO IN BOTH A549 AND 16HBE CELLS AT TIME POINTS WHEN NF- κ B IS ACTIVATED.

It is possible that the levels of intracellular GSH are important in the induction of iNOS, in particular the ratio of GSSG:GSH, since GSSG has been shown to both activate NF- κ B and inhibit its binding to DNA (Droge et al., 1994a; Hodge-Jahngen et al., 1997). I have also shown that decreased GSH levels are concomitant with iNOS induction. I therefore measured the ratio of GSSG to GSH in both A549 and 16HBE cells at 30 minutes, 1 hour, 2 hours, and 4 hours. As mentioned in Materials and Methods, the pH of the samples is important in the assay and adjustment of pH was not possible due to the small volumes of the samples (100 μ l). Data shown below is expressed as the mean of 3 separate experiments but standard errors are absent due to variations within the assay. However a similar trend is shown in each separate experiment. Due to variations I was also unable to measure statistical significance.

Figures 99 and 100 show the effect of treating A549 and 16HBE cells with cytomix, H₂O₂ and BSO for 30 minutes, 1 hour, 2 hours and 4 hours. In both A549 and 16HBE cells cytomix increases the GSSG:GSH ratio at 1 hour, and the trend persists

to 4 hours. H_2O_2 appears to change the ratio of GSSG:GSH in both cell lines at 1 hour, and the trend persists to 4 hours, although the change is much higher at 2 and 4 hours in A549 cells. BSO appears to change the GSSG:GSH ratio at 4 hours in A549 cells and 2 and 4 hours in 16HBE cells.

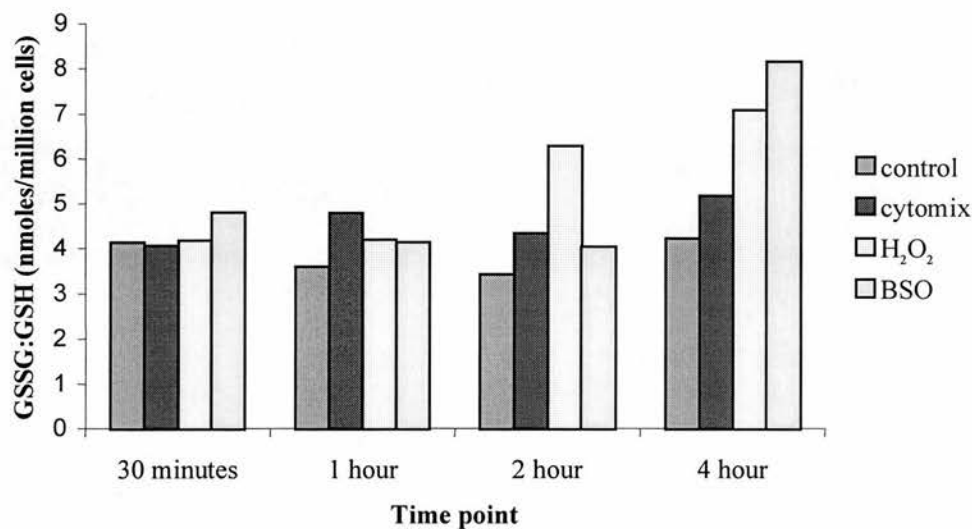


Figure 99: The effect of cytomix, H_2O_2 and BSO on the GSSG:GSH ratio in A549 cells at 30 minutes, 1 hour, 2 hours and 4 hours. Histograms represent the means, and SEMs are absent due to a high level of variation between experiments (n=3).

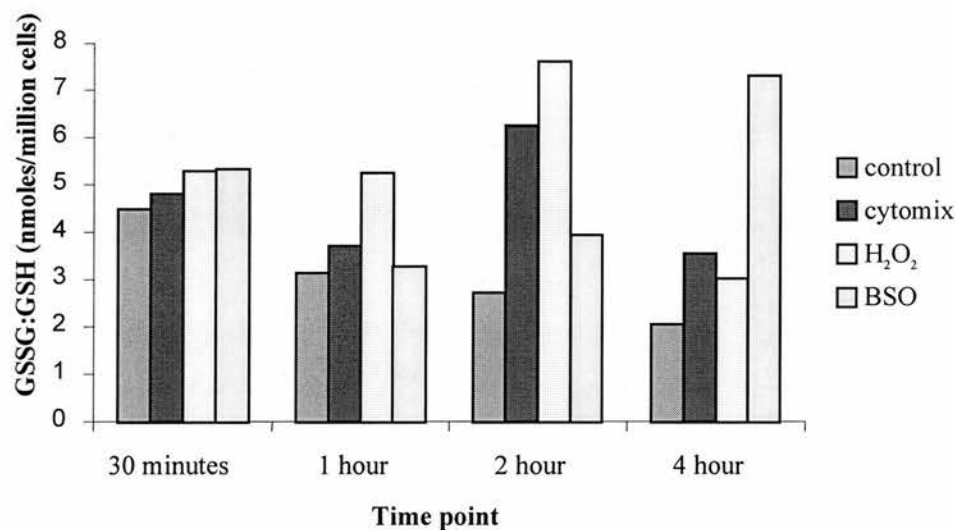


Figure 100: The effect of cytomix, H_2O_2 and BSO on the GSSG:GSH ratio in 16HBE cells at 30 minutes, 1 hour, 2 hours and 4 hours. Histograms represent the means, and SEMs are absent due to a high level of variation between experiments (n=3).

6.8 SUMMARY

- Nuclear binding of NF- κ B is increased, indicating NF- κ B is activated, in both A549 and 16HBE cells at 30 minutes by treatment with cytomix, TNF- α , and IL1- β . IFN- γ did not have any effect on NF- κ B activation. Treatment with LPS for 30 minutes activates NF- κ B in 16HBE cells only.
- NF- κ B is activated in both A549 and 16HBE cells treated with H₂O₂ at 2 hours but not at 30 minutes.
- NF- κ B is activated by BSO at 4 hours in A549 cells and at 2 and 4 hours in 16HBE cells.
- The four thiol compounds, NAC, NAL, GSH, and GSHMEE have no effect on NF- κ B activation at 4 hours in either A549 or 16HBE cells.
- Pre-treatment of both A549 and 16HBE cells with the 4 thiol compounds followed by cytomix treatment for 30 minutes had no effect on cytomix-induced NF- κ B activation.
- Pre-treatment of both A549 and 16HBE cells with thiol compounds followed by H₂O₂ for 2 hours decreased H₂O₂-induced NF- κ B activation.
- Pre-treatment of both A549 and 16HBE cells with thiol compounds followed by TNF- α for 30 minutes had a variable effect on TNF- α -induced NF- κ B activation. A549 cells pre-treated with NAL and GSH decreased TNF- α -induced NF- κ B activation, and 16HBE cells pre-treated with NAL also had decreased TNF- α -induced NF- κ B activation. The other thiol compounds had no effect on TNF- α -induced NF- κ B activation in either cell type.
- Rotenone had no effect on cytomix induced NF- κ B activation in either A549 or 16HBE cells.
- Rotenone had no effect on H₂O₂ induced NF- κ B activation in either A549 or 16HBE cells.
- Rotenone inhibited TNF- α induced NF- κ B activation in both A549 and 16HBE cells.
- The effect of rotenone on TNF- α induction was also observed with iNOS mRNA induction.

6.9 DISCUSSION

Activation of the nuclear transcription factor NF- κ B is considered to be a key element in cellular inflammatory responses and ROS are considered to be important mediators of NF- κ B activation (Marletta, 1993; Matthews et al., 1992; Schreck and Baeuerle, 1994). Oxidant stress (thought to be mediated by H₂O₂ which can function as a signaling molecule in the activation of gene expression (Kretx-Remy et al., 1996)) has been shown to be involved in NF- κ B activation and this activation can often be inhibited by antioxidants (Flohe et al., 1997; Meyer et al., 1993; Sato et al., 1996; Schreck et al., 1991). However, the pathways involved in NF- κ B activation are not fully evaluated and elucidation of the mechanisms involved are likely to be important in the development of strategies for therapeutic intervention in inflammatory diseases including those in the lungs.

The NF- κ B family consists of a variety of proteins (Baldwin, 1996) but the most studied protein is the p50/p65 heterodimer, which is normally present in an inactive form in the cytosol due to its association with the inhibitory protein, I κ B. Activation of NF- κ B involves the phosphorylation and degradation of the I κ B subunit, which masks the DNA binding sites of NF- κ B, enabling NF- κ B to move into the nucleus where it can bind to DNA. NF- κ B activation can be measured by the electrophoretic mobility shift assay (EMSA), which demonstrates NF- κ B binding in the cell nucleus using a radiolabeled oligonucleotide which binds to the DNA binding site of NF- κ B. Demonstration of NF- κ B binding by EMSA often produces 3 bands on the gel and I therefore tried to determine the nature of these bands. This was achieved in 2 ways. The first was to use polyclonal antibodies against the p50 and p65 subunits. This is known as a supershift assay, where binding of the antibodies to the relevant radiolabeled subunit of NF- κ B increases the molecular weight of that subunit and thus retards its progress, or causes it to 'shift' on the gel from its original position. My results show a supershift of the 2 bands thought to be p50 and p65, both antibodies causing a decrease in intensity of the 2 bands which are normally observed. These results indicate that the two top bands (which actually often appear

as one large band) contain the p50 and p65 subunits of NF- κ B. In order to confirm this further, a recombinant I κ B α protein was used. The protein is unlabelled and added to samples prior to addition of the labeled oligonucleotide, and it can therefore bind to NF- κ B, masking the DNA binding site, and prevent the oligonucleotide binding. Addition of the recombinant protein therefore decreases the NF- κ B band observed in activated cells. The results show that addition of the recombinant protein reduces the intensity of the 2 bands produced with cytomix treatment, suggesting that the bands observed are NF- κ B. The use of the I κ B α -protein also suggests that the I κ B α subunit is a likely candidate for inhibition of NF- κ B in these cell lines. The bottom band is thought to be either a product of denaturation or a non-NF- κ B protein since it has been shown by Mr M. Lawson of the Rayne Laboratory to be a non-specific product in a cold displacement assay.

Regulation of NF- κ B involves cascades of phosphorylation and dephosphorylation (Baldwin, 1996; MacKichan et al., 1996). Many enzymes are involved in the pathway leading to NF- κ B activation and may be the target of redox regulation involving oxidants. Most active sites of enzymes contain sulphide bonds, and both oxidising and reducing agents can modify their activity. It has also been suggested that different forms of the inhibitory unit, I κ B, are regulated by different types of kinases, which are likely to play a part in the activation of NF- κ B (Marletta, 1993) and these kinases are also likely to be susceptible to redox regulation. Taking into account the multiple pathways leading to the transcriptional activation of NF- κ B dependent genes, the modulating effects of redox processes are likely to be variable and the pathway for NF- κ B activation may be both cell specific and dependant on the stimulus involved (Flohe et al., 1997). Evidence for the role of ROS in NF- κ B activation arises from the fact that many of the agents that activate NF- κ B, also release ROS or are themselves oxidants (Flohe et al., 1997).

In both the A549 and 16HBE cells cytomix, H₂O₂, TNF- α and IL1- β activated NF- κ B. IFN- γ did not have any obvious effect on NF- κ B activation and LPS induced activation of NF- κ B in 16HBE cells only. The gene for iNOS has been shown to

contain 5 NF- κ B binding sites (Taylor et al., 1998). Binding of NF- κ B to the iNOS gene has previously been observed in A549 cells stimulated with TNF- α and IL1- β , but not IFN- γ (Taylor et al., 1998). NF- κ B activation is considered to be necessary for cytokine induction of iNOS (Amoah-Apraku et al., 1995; Xie et al., 1994) although differences have been observed between murine and human iNOS (Spitsin et al., 1997). Although NF- κ B activation is sufficient for iNOS induction by LPS in murine RAW 264.7 cells, it is not sufficient for iNOS induction in human A549 cells. NF- κ B activation is thus essential, but its activation alone is not necessarily sufficient for iNOS activation, other transcription factors may be necessary in the induction of iNOS (Amoah-Apraku et al., 1995; Spitsin et al., 1997).

ROS have been implicated in the activation of NF- κ B (Flohe et al., 1997; Hecker et al., 1996b; Meyer et al., 1993; Schreck et al., 1991). Antioxidants have been used to prevent ROS induced NF- κ B activation. Therefore the antioxidant status of the cells, such as GSH levels, may be important in modulating NF- κ B activation by ROS.

BSO, as discussed previously, is often used to decrease GSH levels in cells by blocking GSH synthesis. It has been reported (Ginn-Pease and Whisler, 1996), that in Jurkat T cells, pre-treatment with BSO decreased NF- κ B activation by H₂O₂. This suggests that GSH may play a role in preventing NF- κ B activation by oxidants. My results, in contrast, show that in airspace epithelial cells, BSO activates NF- κ B and induces iNOS. Pre-treatment of cells with BSO had no effect on cytomix-induced NO⁻ levels. This suggests that decreasing GSH levels could be involved in NF- κ B activation, so that decreased GSH levels may activate NF- κ B activation. The mechanism may be through a general increase in ROS levels, (which would normally be quenched by GSH) or due to a change in the ratio of GSSG:GSH. A change in the intracellular redox state, particularly that of GSH may result in the modification of sulfhydryl groups present on the cascade of enzymes involved in NF- κ B activation thus causing activation. Changes in the redox state may also inhibit NF- κ B binding to DNA, since the DNA binding site of NF- κ B contains critical cysteine residues which need to be in a reduced state for binding to occur.

Antioxidants, particularly thiol containing compounds, are in widespread use to prevent activation of transcription factors such as NF- κ B, and thus inhibition of the release of inflammatory mediators such as cytokines and NO \cdot (Flohe et al., 1997; Hecker et al., 1996a; Hecker et al., 1996b; Meyer et al., 1993; Sato et al., 1996; Schreck et al., 1991). NAC and NAL are potent scavenger's of the hydroxyl radical (Vanderbist et al., 1996) and NAC has been shown to protect against the toxic effects of H₂O₂ (Lyons et al., 1992; Mulier et al., 1998). Thiol containing antioxidants are also able to increase intracellular GSH levels. Different antioxidants have, however, been shown to have different effects on iNOS induction at both pre and post-transcriptional levels (Hecker et al., 1996b). It has been reported (Hecker et al., 1996b) that in vascular smooth muscle cells, although a variety of antioxidants are able to inhibit superoxide production by Phorbol 12-myristate 13-acetate (PMA), indicating they are working efficiently within the cell, they don't necessarily inhibit iNOS induction by IL1- β , suggesting that ROS are not necessary for cytokine induced NF- κ B activation and iNOS induction.

I examined the effects of thiol antioxidants on NF- κ B activation (the 4 antioxidants themselves having no effect on NF- κ B activation (data not shown)) by cytomix, TNF- α and H₂O₂. Both A549 and 16HBE cells were pre-treated with the 4 thiol antioxidants NAC, NAL, GSH, and GSHMEE for 4 hours, the time point at which intracellular GSH levels are increased. The cells were then washed thoroughly (to avoid scavenging of ROS) and treated with cytomix, TNF- α or H₂O₂. The data show that pre-treatment of both A549 and 16HBE cells with the thiol compounds followed by cytomix for 30 minutes had no effect on cytomix induced NF- κ B activation. This suggests that increasing GSH by these compounds does not effect NF- κ B activation by cytokines. Pre-treatment of the cells with the thiol compounds followed by H₂O₂ for 2 hours did, however, decrease H₂O₂ induced NF- κ B activation, suggesting that oxidant-induced NF- κ B activation can be inhibited by increasing GSH levels. Pre-treatment of the cells with thiol compounds followed by TNF- α for 30 minutes, gave variable results. Pre-treatment of A549 cells with NAL and GSH decreased NF- κ B activation compared to TNF- α alone. A similar result was observed in 16HBE cells

pre-treated with NAL. The result shown with TNF- α was surprising since TNF- α is known to increase superoxide anion release from the mitochondria and increased GSH levels would therefore likely inhibit NF- κ B activation by TNF- α . The results observed may therefore be explained in 2 ways. One is that the level of TNF- α used to activate NF- κ B was too high, and partial protection may be observed with lower levels of TNF- α . Since results show that there is a huge increase in NF- κ B activation with 10ng/ml of TNF- α , I may not be able to observe any partial inhibition that is occurring. The second explanation is that although TNF- α releases oxidants that activate NF- κ B, it may also activate a separate pathway that does not involve oxidants.

Rotenone was therefore used to distinguish between these two theories. Rotenone is an inhibitor of complex 1 of the mitochondrial electron transport chain and prevents superoxide anion release upon stimulation by compounds such as TNF- α .

Cells were pre-treated for 30 minutes with rotenone followed by cytomix or TNF- α for 30 minutes, or H₂O₂ for 2 hours. A549 cells treated with rotenone followed by cytomix or H₂O₂ showed no decrease in NF- κ B activation following either stimuli. This result was also observed in 16HBE cells. In both cell types, cells pre-treated with rotenone followed by TNF- α , had reduced activation of NF- κ B by TNF- α . This suggests that TNF- α may activate NF- κ B by increasing ROS release from the mitochondria. INOS mRNA expression was also assessed following pre-treatment with rotenone followed by cytomix, TNF- α and H₂O₂. Results were only obtained for the A549 cells however, since rotenone and chloroform caused cell death in the 16HBE cells at 4 hours. Using RT-PCR, pre-treatment of cells with rotenone had no effect on cytomix or H₂O₂ induced iNOS levels, but inhibited TNF- α induced iNOS. Thus it is likely that TNF- α activates NF- κ B and induces NO by an oxidant-mediated pathway. It is therefore likely that inhibition of TNF- α induced NF- κ B activation by increased GSH levels is not observed with all compounds due to the dose of TNF- α being too high and the increase in intracellular GSH by the antioxidants may not be sufficient to reduce the amount of ROS produced by TNF- α .

A direct oxidant (H_2O_2) mediated pathway of NF- κ B activation can therefore be blocked by increasing GSH levels with thiol antioxidants, but increasing GSH levels has no effect on cytomix induced NF- κ B activation, suggesting that cytomix may activate NF- κ B by a different pathway. This is further supported by results observed with rotenone. Rotenone had no effect on cytomix induced NF- κ B and iNOS induction, suggesting that production of oxidants by cytomix do not play an essential role in cytokine induced NF- κ B activation.

Although it is widely accepted that NF- κ B is activated by ROS and that this activation can be inhibited by antioxidants, particularly thiol-containing antioxidants, there is growing controversy that more than one pathway exists in the activation of NF- κ B. In support of the data presented herein there are now several reports showing evidence that NF- κ B may be activated by a pathway that is unaffected by antioxidants and may not rely on oxidants.

In studies of NF- κ B activation by ROS in Jurkat T cells, KB epidermal cells, and EL4.NOB-1 T cells, (Brennan and O'Neill, 1994) both H_2O_2 and TNF- α activated NF- κ B in Jurkat T cells and this activation was inhibited by NAC. However, NAC did not offer protection against H_2O_2 and TNF- α in the other two cells types. This suggests that the ROS model of NF- κ B activation is dependant on the cell type and may not be necessary for NF- κ B activation by TNF- α . Further evidence against the hypothesis that ROS are always necessary for the activation of NF- κ B has been reported by MacKichie *et al* (MacKichie et al., 1996), who found that the p105 Rel protein, which contains the p50 subunit of NF- κ B and the I κ B inhibitor, requires phosphorylation for proteolytic processing into the p50 and I κ B subunits, and hence NF- κ B activation. Compounds that block I κ B phosphorylation and degradation, also block phosphorylation of the p105 protein, but antioxidants do not block phosphorylation, suggesting that a redox insensitive pathway may exist. The insensitivity of the p105 protein to antioxidants demonstrates that each I κ B inhibitor is independently regulated, and the response induced reflects the specific role of the

inhibitor in determining the response of NF- κ B to stimuli (MacKichan et al., 1996). Suzuki *et al* (Suzuki et al., 1994) reported that 2 activators of NF- κ B, calyculin A and okadaic acid, were not inhibited by NAC, even though calyculin A was inhibited by rotenone (suggesting oxidant involvement). Both calyculin A and okadaic acid are phosphatase inhibitors and therefore target signal transduction. It is thought that they may either target the pathway downstream from an antioxidant sensitive step or work via a different signaling pathway, since inhibition of phosphatases is not a step in TNF- α induced NF- κ B activation (Suzuki et al., 1994).

Thus it is possible that cytomix and H₂O₂ activate NF- κ B via different pathways. H₂O₂ by an oxidant-induced pathway and cytomix by a signaling pathway independent of oxidants. TNF- α produces oxidants from the mitochondria and this may enhance cytomix induced NF- κ B activation but is probably not essential for activation.

Although there are many reports suggesting that ROS are involved in NF- κ B activation and that antioxidants can inhibit this activation, few have focused on the role of the redox state of the cell, in particular GSH.

Thioredoxin (TRX) is an important cellular protein with antioxidant properties and is important in NF- κ B activation since it is involved in the binding of NF- κ B to DNA (Hayashi et al., 1993). It is possible that redox regulation of NF- κ B by TRX occurs at a point after the dissociation of I κ B from NF- κ B. The DNA binding motif of NF- κ B contains cysteine residues which need to be reduced for DNA binding of NF- κ B to occur, and TRX ensures that they remain reduced. Thus a redox mechanism exists, mediated by TRX which regulates NF- κ B-mediated gene expression (Hayashi et al., 1993). GSSG, which is formed by oxidation of GSH, acts as an oxidant and it is possible that levels of GSSG play an important role in preventing the activation of NF- κ B, by competing with TRX for the cysteine residues, oxidising them and ensuring NF- κ B does not bind to DNA. Droge *et al* (Droge et al., 1994) reported that low levels of GSSG prevented NF- κ B activation

where as too high levels of GSSG inhibited DNA binding of NF- κ B and effects of GSSG were antagonised by TRX. NF- κ B is thought to be activated by a cascade of membrane bound and cytoplasmic protein kinases including tyrosine kinases of the src family, 2 of which are activated by H₂O₂. GSSG may play a role in stabilizing phosphorylation of the kinases, (and thus activation of NF- κ B), by oxidising the active sites of phosphatases and therefore inhibiting their phosphatase activity. Evidence that GSSG is involved in NF- κ B activation is also shown by the fact that a potent inducer of NF- κ B teradecanoylphorbolacetate (TPA) is associated with increased GSSG levels and a change in the GSSG:GSH ratio. Droge *et al* (Droge et al., 1994) reported that BSO inhibited TPA activation of NF- κ B, and decreased GSSG levels. BCNU, a compound that inhibits glutathione reductase and therefore increases GSSG levels, increased TPA induced activation of NF- κ B. It is therefore possible that GSSG levels are important in NF- κ B activation and that H₂O₂ or oxidants activate NF- κ B by altering the redox state of the cell, in particular the level of GSSG. However high levels of GSSG can inhibit NF- κ B binding to DNA, and this inhibition occurs even in the presence of an excess of thiols, and only by increasing levels of TRX is this inhibition overcome (Droge et al., 1994a). Cells containing higher levels of TRX also require increased levels of GSSG for activation of NF- κ B producing further evidence that GSSG is involved. DNA binding of NF- κ B can be inhibited by thiol modulating agents and enhanced by reducing agents as shown by mutating cysteine residues in the binding site (Matthews et al., 1992). Thus it is likely that an optimal level of GSSG is required for NF- κ B activation, and this is likely to occur during an oxidant stress (Droge et al., 1994; Galter et al., 1997).

I therefore measured the levels of GSSG in cells treated with cytomix, H₂O₂ and BSO at various time points ranging from 30 minutes to 4 hours, and determined if the ratio of GSSG:GSH had any effect on NF- κ B activation. The data show that the ratio of GSSG:GSH increases with cytomix at 1 and 2 hours in both cell types. Although TNF- α increases ROS levels at less than 30 minutes, it is unlikely that the GSSG:GSH ratio is involved in cytomix induced NF- κ B activation, since the ratio changed at a time point after NF- κ B activation occurs. The increase observed at

these later time points (1-4 hours) is probably due to the increased ROS levels caused by TNF- α . This change in the ratio may contribute to NF- κ B activation but is probably not essential to cytomix-mediated NF- κ B activation. H₂O₂, however, produced an increased GSSG:GSH ratio at 1 and 2 hours, and this may be important in the activation of NF- κ B by ROS. Likewise, BSO produces an increased GSSG:GSH ratio at time points when NF- κ B activation occurs (4 hours in A549 cells and 2 hours in 16HBE cells). It is therefore possible that oxidant mediated activation of NF- κ B is due, at least in part, to the levels of GSSG, in particular the ratio of GSSG:GSH, which activates NF- κ B at lower levels, and inhibits activation at higher levels. Inhibition of NF- κ B activation by high levels of GSSG is observed with BSO, which increases the ratio of GSSG:GSH at 24 hours by a much larger amount (data not shown) than that observed at 4 hours but NF- κ B activity is not observed at 24 hours. The change in ratio at these time points is also likely to be the cause for the decrease in GSH observed with the treatments at 24 hours.

In conclusion, NF- κ B is activated by stimuli that induce NOS but data suggests that NF- κ B is activated by 2 pathways, one an oxidant mediated pathway, which can be inhibited by thiol-containing antioxidants that increase intracellular GSH levels. This activation is also associated with a shift in the ratio of GSSG:GSH. The other pathway appears to be independent of oxidants in that thiol-containing antioxidants and increased GSH levels have no effect on NF- κ B activation and the ratio of GSSG:GSH is not associated with NF- κ B activation. Thus it is likely that NF- κ B activation, and hence NOS induction can be selectively inhibited under different conditions.

7 Final Discussion

7.1 SUMMARY

- Intracellular GSH is relatively stable in both A549 and 16HBE cells at between 70 and 99% confluency in the presence of serum.
- NO[•] is induced by cytomix, TNF- α , and H₂O₂ in both A549 and 16HBE cells, and is also induced by LPS in 16HBE cells as measured by both nitrite production and iNOS mRNA levels.
- This induction is concomitant with a decrease in GSH levels in both A549 and 16HBE cells.
- This induction is also concomitant with NF- κ B activation, as measured by the electromobility shift assay in both A549 and 16HBE cells.
- BSO causes a decrease in GSH levels, which is both accompanied by an increase in NO[•] release, iNOS mRNA and NF- κ B activation in both A549 and 16HBE cells.
- The thiol compounds NAC, NAL, GSH, and GSHMEE cause a significant increase in GSH at 4 hours in both A549 and 16HBE cells.
- Pre-treatment of cells with thiol compounds followed by cytomix had no effect on cytomix-induced NO[•] release, iNOS mRNA or NF- κ B activation in both A549 and 16HBE cells.
- Pre-treatment of cells with thiol compounds followed by H₂O₂ resulted in a decrease in H₂O₂-induced NO[•] release, iNOS mRNA and NF- κ B activation in both A549 and 16HBE cells.
- Pre-treatment of cells with thiol compounds followed by TNF- α had no effect on TNF- α -induced NF- κ B activation in both A549 and 16HBE cells.
- Rotenone inhibited TNF- α induced NF- κ B activation but had no effect on cytomix or H₂O₂ induced activation in A549 or 16HBE cells. This effect was also observed in A549 iNOS mRNA levels.
- The ratio of GSSG:GSH is increased in cells following treatment with cytomix, H₂O₂ and BSO. The ratio is increased in both H₂O₂ and BSO treated cells at time

points when NF- κ B activation occurs, but occurs after the time at which cytomix induces NF- κ B activation.

- There are no obvious differences between the two cell types used, the A549 cell line and the 16HBE cell line, other than the effects observed with LPS in 16HBE cells.

7.2 CONCLUSIONS

I should emphasise that the use of cell lines are a very useful tool in investigating the metabolic and molecular pathways of individual cells but they cannot mimic the complexities of the situation *in vivo* where interactions between cell types, metabolic products and the environment occur. The use of cell lines, however, allows the investigation to focus on individual mechanisms, *in vitro*, (Gille and Joenje, 1992). The data obtained will therefore give insight into pathways which need to be elucidated in order to understand certain pathologic conditions but results need to be validated both in primary cell cultures and *in vivo*. Harvesting of cells from the body leads to changes within the cells, such as antioxidants whose levels change within 30 minutes of removal of type II cells from the lung (Kinnula et al., 1992), and thus, even in primary cultures, the antioxidant response is likely to be different from that seen *in vivo*. Transformation of cells in order to facilitate their growth is also likely to change some cellular characteristics. The cells used herein, however, do retain many of the characteristics of the original cell and since there are no obvious differences between cell lines, results will hopefully resemble those *in vivo*.

Are intracellular GSH levels affected by culture conditions, and if so when are they relatively stable?

Intracellular GSH levels were measured in both A549 and 16HBE cells at various confluencies. The cells were seeded at low densities in medium complemented with 10% serum. GSH was found to increase as cells proliferated. A relatively stable level of GSH was obtained from 70% to 100% confluency in both cell types, GSH decreased once cells had formed a confluent monolayer. Cells grown in serum free

medium had lower GSH levels than those grown in serum supplemented medium, and therefore cells were treated at 70% confluency in serum supplemented medium.

Can NO[•] be induced in both A549 and 16HBE cells with cytomix, individual cytokines, LPS and H₂O₂?

NO[•] was successfully induced at 24 hours in both cell lines following cyomix, TNF- α and H₂O₂. LPS has no effect in A549 cells, but increased NO[•] levels in 16HBE cells. mRNA levels for iNOS were also observed in both cell types, and iNOS was switched on in both cell types following cytomix, TNF- α , H₂O₂, and LPS in 16HBE cells. Cells stimulated with increased cytomix levels (20ng/ml) or with co-incubation of cytomix and H₂O₂ did not show increased levels of NO[•] release compared to cytomix, 10ng/ml. This is possibly due to negative feedback by NO[•] itself or due to the top of the concentration response being reached.

Does NO[•] induction affect intracellular GSH levels?

Intracellular GSH levels were measured at 24 hours, the time point at which NO was released from the cell. An increase in NO was concomitant with a decrease in intracellular GSH levels. The decrease in GSH was not, however, due to NO[•] formation, since inhibitors of NO[•] did not prevent the decrease in GSH. Decreased GSH levels were therefore likely to be due to the effects of the stimuli themselves, such as TNF- α , and H₂O₂. TNF- α causes the release of superoxide anions from the mitochondria, which are converted to H₂O₂ by SOD in the cytosol. H₂O₂, the likely ROS involved in NF- κ B activation, (Kretx-Remy et al., 1996) and hence NO[•] induction is broken down by GPx and GSH to form GSSG and this is likely to result in a decrease in intracellular GSH since GSSG is exported from the cells.

Does decreasing intracellular GSH affect NO[•] induction?

BSO was used to decrease GSH since GSH is thought to be involved in NO[•] induction. I found that decreasing GSH with BSO resulted in the induction of iNOS and NO release. The increase in NO[•] following addition of BSO is likely to be due to an increase in the intracellular ROS concentrations. ROS are released from cellular reactions and organelles and are normally effectively quenched by GSH. However if GSH levels are decreased, then these oxidants may activate NF- κ B and hence induce iNOS and NO[•] release.

Does increasing GSH affect NO[•] induction?

The four thiol compounds NAC, NAL, GSH, and GSHMEE were used to increase GSH levels in both A549 and 16HBE cells. Cells were then pre-treated with the 4 thiol compounds followed by cytomix and H₂O₂. Pre-treatment of cells with thiol compounds followed by cytomix had no effect on cytomix induced NO[•] induction or iNOS mRNA levels, suggesting that the redox status of the cell, in particular that of GSH is not involved in the induction of iNOS by cytomix. Pre-treatment of cells with thiol compounds followed by H₂O₂ however, decreased H₂O₂ induced NO[•] induction and iNOS mRNA levels, suggesting that GSH is involved in oxidant induced NO[•]. GSH may therefore be acting as an antioxidant, scavenging ROS, or may be involved directly in the induction of NO[•].

Is NF- κ B activated in cells treated with cytomix, individual cytokines, LPS and H₂O₂?

NF- κ B is activated in both A549 and 16HBE cells following cytomix, TNF- α , IL1- β and H₂O₂. IFN- γ had no obvious effect on NF- κ B activation and LPS activates NF- κ B in 16HBE cells only. Activation occurs at 30 minutes for cytokines and LPS and 2 hours for H₂O₂. H₂O₂ may take longer than cytomix since it is possible that NF- κ B activation by H₂O₂ requires changes in the redox state of the

cell, whereas cytokines activate NF- κ B independently of the redox state of the cells. It is possible that H₂O₂ activation of NF- κ B requires a shift in the GSSG:GSH ratio.

Does increasing intracellular GSH levels affect cytomix, TNF- α and H₂O₂ induction of NF- κ B activation?

Pre-treatment of both cell types with thiol compounds, followed by cytomix had no effect on cytomix induced NF- κ B activation. It did however decrease H₂O₂ induced activation of NF- κ B and had a varied effect on TNF- α induced activation. This suggests that increased GSH levels can prevent oxidant induced NF- κ B activation. TNF- α works via increasing ROS within the cell. Thus NF- κ B activation by TNF- α would therefore be expected to be decreased by increased GSH levels, but it was only partially decreased by some thiol compounds, and not at all by other thiol compounds. To determine if TNF- α was having its effect via oxidant induced activation, I examined the effects of rotenone on TNF- α , cytomix and H₂O₂ induced NF- κ B activation and iNOS mRNA induction. Rotenone inhibits complex I of the electron transport chain, and therefore prevents ROS release from the mitochondria. Cells pre-treated with rotenone followed by cytomix and H₂O₂ had no effect on cytomix or H₂O₂ induced NF- κ B activation, suggesting that H₂O₂ is working as a direct oxidant, independent of the mitochondria, and cytomix is working via a pathway that does not rely on increased ROS for NF- κ B activation. Cells pre-treated with rotenone followed by TNF- α , however, showed reduced levels of NF- κ B activation, compared to TNF- α alone, suggesting that TNF- α is working via an oxidant mediated pathway. This data was further supported by similar observations on TNF- α -induced iNOS mRNA levels in rotenone treated A549 cells. The dose of TNF- α used was 10ng/ml which produced a huge increase in the activation of NF- κ B. The increase in GSH levels produced by the thiol compounds may therefore not be completely sufficient in blocking ROS released by TNF- α and hence only partially prevent NF- κ B activation. TNF- α induced NF- κ B activation using lower doses of TNF- α may be inhibited by increased GSH levels. Since rotenone had no

effect on cytomix induced NF- κ B activation or iNOS induction, it can be assumed that cytomix is not working via superoxide anion production from the mitochondria and hence its concentration is not important in protection studies.

Does the GSSG:GSH ratio affect NF- κ B activation by cytokines, H₂O₂ and BSO?

GSSG levels were measured in both A549 and 16HBE cells between 30 minutes and 4 hours in order to determine if the ratio of GSSG:GSH was increased at the time of NF- κ B activation. Cytomix activates NF- κ B at 30 minutes and there is not a large difference in the GSSG:GSH ratio in either cell type at 30 minutes, suggesting cytomix is not working via a mechanism which depends on the GSSG:GSH ratio. H₂O₂, however, produced an increased GSSG:GSH ratio at 1 hour, continuing to 4 hours, suggesting that an increase in the GSSG:GSH ratio may play a part in H₂O₂ induction. Activation of NF- κ B was not measured at 1 hour, but H₂O₂ increased NF- κ B activation at 2 hours, a time point at which the GSSG:GSH ratio was high in both cell types. BSO increases the GSSG:GSH levels at 4 hours in A549 cells and 2 hours in the 16HBE cells, and this increase continues to 24 hours (data not shown), and is likely to be involved in BSO activation of NF- κ B and iNOS induction. The ratio of GSSG:GSH in cytomix and H₂O₂ treated cells returns to that of control at 24 hours, suggesting that the decrease in GSH observed at 24 hours is due to the oxidation of GSSG:GSH at earlier time points, and an efflux of GSSG from the cell as it accumulates.

7.3 TO CONCLUDE:

Both A549 and 16HBE cells were treated in the same manner throughout the thesis and there were no obvious differences between the cells in these experiments, other than the effect of LPS on 16HBE cells. It has been reported (Amoah-Apraku et al., 1995) that LPS is only a weak inducer of NF- κ B in non-immune cells, and it is insufficient to activate NF- κ B and iNOS in human A549 cells, (Spitsin et al., 1997)

which require a mixture of cytokines. 16HBE cells are therefore more susceptible to LPS, and bronchial epithelial cells may therefore play a bigger role in the immune response *in vivo*.

NO[•] is induced in both A549 and 16HBE cells by cytomix, TNF- α , H₂O₂ and BSO and this increase is concomitant with a decrease in GSH. The decrease in GSH is not, however dependent on NO[•] release, but is likely to be caused by an increased oxidant burden which alters the GSSG:GSH ratio. Both H₂O₂ and BSO increase the GSSG:GSH ratio at the time point at which they activate NF- κ B, suggesting that the GSSG:GSH ratio may play a part in oxidant induced NF- κ B activation and iNOS induction. Cytomix, however, is likely to activate NF- κ B and induce iNOS by a different pathway, which may or may not involve oxidants, since the ratio of GSSG:GSH is not increased at the time point at which NF- κ B is activated. This is supported by the data using the thiol compounds to increase GSH levels. Increased GSH levels can reduce H₂O₂ induced NF- κ B activation and NO[•] release, they cannot, however reduce cytokine induced NF- κ B activation or NO[•] release. Thus separate pathways are likely to exist in the induction of NO[•], and further investigation into these pathways may provide a mechanism for selectively inhibiting NO[•] induction during inflammation. During inflammation NO[•] can be induced in large amounts by NF- κ B activation, and may itself cause lung damage due to the formation of peroxynitrite (Ignarro and Murad, 1995; Xie et al., 1994). Peroxynitrite, is formed in large amounts during inflammation due to the availability of ROS and NO[•] and inhibition of its formation may be beneficial in preventing lung damage such as that observed in asthma, COPD, ARDS and emphysema (Kharitonov et al., 1996; Kooy et al., 1995; Royall.JA. and Kooy, 1997; Saleh et al., 1997).

7.4 FUTURE STUDIES

- Since cell lines were used throughout this thesis it would be interesting to carry out the key experiments in primary cell cultures, to determine if the effects observed in this thesis also occur in primary culture. This would indicate that the

pathways determined herein are also the pathways that occur *in vivo* and may therefore provide insight into mechanisms for modulating the activation of NF- κ B and NO \cdot induction selectively in inflammatory conditions of the lungs. It would therefore be ideal to:

- ◆ Investigate the effects of cytomix, H₂O₂ and TNF- α on NO \cdot induction in primary cell cultures and to examine the effect of NO induction on GSH levels.
 - ◆ Investigate the effects of cytomix, H₂O₂ and TNF- α on NF- κ B activation in primary cell cultures, and to determine if increased GSH levels prevent NF- κ B induced activation.
- Since it is likely that GSH and GSSG levels are important in NF- κ B activation, and possibly are more important than ROS themselves, future studies should examine the effect of changing intracellular GSSG levels and the GSH:GSSG ratio by compounds such as BCNU, and determine the effect of altered GSSG levels on cytomix and H₂O₂ induced NF- κ B activation. The assay used herein for measuring GSSG levels does not seem to be sensitive enough for accurate measurements, although results show a trend throughout, statistical analysis was not possible. It would therefore be beneficial to either find a different assay for GSSG measurement, or to increase the volumes used from 100 μ l to a few millilitres, which would enable the adjustment of the pH of the samples.
 - NF- κ B has been shown to be necessary in the induction of iNOS and NO \cdot but not necessarily sufficient in some circumstances. Further studies should therefore investigate:
 - ◆ Other transcription factors such as IRF-1, the interferon regulatory factor, which is activated by IFN- γ , and will possibly give insight into a non-oxidative pathway induced by cytomix. Since IFN- γ does not have a large effect on NF- κ B activation itself, it is likely to be working in a synergistic manner with TNF- α and IL1- β in the induction of NO \cdot .

Different parts of the pathway involved in iNOS induction should be examined such as:

- ◆ I κ B levels, degradation indicating NF- κ B activation, and comparison of I κ B levels to GSH and GSSG levels, in particular the GSH:GSSG ratio.
- ◆ Investigation of kinase phosphorylation which is implicated in the activation of NF- κ B in order to determine if varying levels of GSSG affects phosphorylation and NF- κ B activation.
- To determine an alternative method for dissolving rotenone (such as DMSO) to ensure future experiments using rotenone do not result in cell death.

Reference List

Adcock, I.M., Brown, C.R., Ojung, K., and Barnes, P.J. (1994). Oxidative Stress Induces NF Kappa B DNA Binding and Inducible NOS mRNA in Human Epithelial Cells. *Biochem.Biophys.Res.Comm.* 199, 1518-1524.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1989). Cell Growth and Division. *Molecular Biology of the Cell*. New York: Garland, pp. 727-790.

Amoah-Apraku, B., Chandler, L.J., Harrison, J.K., Tang, S.S., Inglefinger, J.R., and Guzman, N.J. (1995). NF- κ B and Transcriptional Control of Renal Epithelial-Inducible Nitric Oxide Synthase. *Kidney Int.* 48, 674-682.

Anderson, M.E., Powrie, F., Puri, R.N., and Meister, A. (1985). Glutathione Monoethyl Ester: Preparation, Uptake by Tissues, and Conversion to Glutathione. *Arch.Biochem.Biophys.* 239, 538-548.

Andrew, N.C. and Faller, D.V. (1991). A Rapid Micropreparation Technique for Extraction of DNA-binding Proteins from Limiting Numbers of Mammalian Cells. *Nuc.Acids.Res.* 19, 2499-2504.

Anggard, E. (1994). Nitric Oxide: Mediator, Murderer, and Medicine. *Lancet* 343, 1199-1206.

Arouma, O., Halliwell, B., Hoey, B.M., and Butler, J. (1989). The Antioxidant Action of N-acetylcysteine: Its Reaction with Hydrogen Peroxide, Hydroxyl Radical, Superoxide and Hypochlorous Acid. *Free Rad Biol Med* 6, 593-597.

Ashbaugh, D.G., Bigelow, D.B., Petty, T.L., and Levine, B.E. (1967). Acute Respiratory Distress in Adults. *Lancet* 2, 319-323.

Assreuy, J., Cunha, FQ., Liew, FY., and Moncada, S. (1993). Feedback Inhibition of Nitric Oxide Synthase Activity by Nitric Oxide. *Br.J.Pharmacol.* 108, 833-837.

Atzori, L., Dypbunkt, J.M., Hybbinette, S., Moldeus, P., and Grafstrom, R.C. (1994). Modifications of Cellular Thiols during Growth and Squamous Differentiation of Cultured Human Bronchial Epithelial Cells. *Exp.Cell Res.* 211, 115-120.

Azzawi, M., Bradley, B., Jeffrey, PK., Frew, A., Wardlaw, AJ., Knowles, G., Assoui, B., Collins, JV., Durham, S., and Kay, AB. (1990). Identification of Activated T Lymphocytes and Eosinophils in Bronchial Biopsies in Stable Atopic Asthma. *Am Rev Respir Dis* 142, 1407-1413.

Azzawi, M., Johnston, PW., Kay, AB., and Jeffrey, PK. (1992). T Lymphocytes and Activated Eosinophils in Airway Mucosa in Fatal Asthma and Cystic Fibrosis. *Am Rev Respir Dis* 145, 1477-1482.

Baeuerle, PA. and Baichwal, VR. (1997). NF- κ B as a Frequent Target for Immunosuppressive and Anti-Inflammatory Molecules. *Advances in Immunology.* 65, 111-137.

Baeuerle, PA., Rupec, RA., and Pahl, HL. (1996). Reactive Oxygen Intermediates as Second Messengers of a General Pathogen Response. *Pathologie Biologie* 44, 29-35.

Baldwin, AS. (1996). The NF- κ B and I κ B Proteins: New Discoveries and Insights. *Ann Rev Immunol* 14, 649-681.

Balfour-Lynn, IM., Lavery, A., and Dinwiddie, R. (1996). Reduced Upper Airway Nitric Oxide in Cystic Fibrosis. *Arch.Dis.Childhood.* 75, 319-322.

Barnes, P.J. (1993). Nitric Oxide and Airways. *Eur Respir J* 6, 163-165.

Barnes, P.J. (1996). Pathophysiology of Asthma. *Brit.J.Clin.Pharm.* 42, 3-10.

Barnes, P.J. (1990). Reactive Oxygen Species and Airway Inflammation. *Free Rad Biol Med* 9, 235-243.

Barnes, P.J. and Belvisi, M.G. (1993). Nitric Oxide and Lung Disease. *Thorax* 48, 1034-1043.

Barnes, P.J. and Liew, F.Y. (1995). Nitric Oxide and Asthmatic Inflammation. *Imm.Today* 16, 128-130.

Bast, A., Guido, R.M., Haenen, M., and Doelman, C. (1991). Oxidants and Antioxidants: State of the Art. *Am.J.Med.* 91 suppl3C, 3C-3S-3C-53S.

Beasley, R., Roche, W., Roberts, J., and Holgate, S. (1985). Cellular Events in the Bronchi in Mild Asthma and after Bronchial Provocation. *Am Rev Respir Dis* 139, 599-606.

Beckman, J.S., Beckman, T.W., Chen, J., Marshal, P.A., and Freeman, B.A. (1990). Apparent Hydroxyl Radical Production by Peroxynitrite: Implications for Endothelial Injury from Nitric Oxide and Superoxide. *Proc.Natl.Acad.Sci.USA* 87, 1620-1624.

Blackwelll, T.S., Blackwell, T.R., Holden, E.P., Christman, B.W., and Christman, J.W. (1996). In Vivo Antioxidant Treatment Suppresses Nuclear Factor- κ B Activation and Neutrophilic Lung Inflammation. *J.Immunol.* 157, 1630-1637.

Brennan, P. and O'Neill, L. (1994). Effects of Oxidants and Antioxidants on Nuclear Factor κ B Activation in Three Different Cell Lines: Evidence Against a Universal Hypothesis Involving Oxygen Radicals. *Biochem.Biophys.Acta* 1260, 167-175.

Brigham, K.L. (1990). Oxidant Stress and Adult Respiratory Distress Syndrome. *Eur Respir J* 3 suppl. 11, 482s-484s.

Bunnet, E. and Pacht, ER. (1993). Oxidized Glutathione is Increased in the Alveolar fluid of Patients with the Adult Respiratory Distress Syndrome. *Am Rev Respir Dis* 148(5), 1174-1178.

Burrowski, DM., Deneke, SM., Lawrence, RA., and Jenkinson, SG. (1995). A Non-inducible Cystine Transport System in Rat Alveolar Type II Cells. *Am J Physiol* 268, L21-L26

Busse, W., Calhoun, W., and Sedgwick, J. (1993). Mechanism of Airway Inflammation in Asthma. *Am Rev Respir Dis* 147, s20-s24

Byrnes, CA., Dinarevic, S., Shinebourne, EA., Barnes, P.J., and Bush, A. (1997). Exhaled Nitric Oxide Measurements in Normal and Asthmatic Children. *Pediatric Pulmonology*. 24, 312-318.

Calhoun, W., Reed, HE., Moest, DR., and Stevens, CA. (1992). Enhanced Superoxide Production by Alveolar Macrophages and Air-Space Cells, Airway Inflammation, and Alveolar Macrophage Density Changes after Segmental Antigen Bronchoprovocation in Allergic Subjects. *Am Rev Respir Dis* 145, 317-325.

Cantin, AM. and Begin, R. (1991). Glutathione and Inflammatory Disorders of the Lung. *Lung* 169, 123-138.

Cantin, AM., North, SL., Hubbard, RC., and Crystal, RG. (1987). Normal Alveolar Epithelial Lining Fluid Contains High Levels of Glutathione. *J.Appl.Physiol.* 63, 152-157.

Chartrain, NA., Geller, DA., Koty, PP., Sitrin, NF., Nussler, AK., Hoffman, EP., Billiar, TR., Hutchinson, NI., and Mudgett, JS. (1994). Molecular Cloning, Structure, and Chromosomal Localization of the Human Inducible Nitric Oxide Synthase Gene. *J.Biol.Chem.* 269, 6755-6772.

Chen, S., Wu, H., Jen, C., Wing, L., Lei, H., Tsao, C., and Chang, W. (1992). Cytoprotective Effect of Reduced Glutathione in Hydrogen Peroxide-induced Endothelial Cell Injury. *Prostaglandins Leukotrienes and Essential Fatty Acids.* 45, 299-305.

Chesrown, SE., Monnier, J., Visner, G., and Nick, HS. (1994). Regulation of Inducible Nitric Oxide Synthase mRNA levels by LPS, IFN- γ , TGF- β and IL-10 in Murine Macrophage Cell lines and Rat Peritoneal Macrophages. *Biochem.Biophys.Res.Comm.* 200, 126-134.

Chinard, FP. (1998). Priestly and Lavoisier: Oxygen and Carbon Dioxide. in: Proctar, D.F. (Ed.) *A History of Breathing Physiology*, 83rd edn. *Lung Biology in Health and Disease.* 203-221.

Clancy, RM., Leszczunska-Piziak, J., and Abramson, SB. (1992). Nitric Oxide, an Endothelial Cell Relaxation Factor, Inhibits Neutrophil Superoxide Anion Production via a Direct Action on the NADPH Oxidase. *J.Clin.Invest.* 90, 1116-1121.

Cochrane, CG. (1991). Cellular Injury by Oxidants. *Am.J.Med.* 91, 23s-30s.

Cochrane, CG., Spragg, R., and Revak, SD. (1983). Pathogenesis of the Adult Respiratory Distress Syndrome: Evidence of Oxidant Activity in Bronchoalveolar Lavage Fluid. *Am J Physiol* 71, 754-761.

Cozens, AL., Yezzi, MJ., Kunzelmann, K., Ohnishi, T., Chin, L., Eng, K., Finkbeiner, WE., Widdicombe, JH., and Gruenert, DC. (1994). CFTR Expression and Chloride

Secretion in Polarized Immortal Human Bronchial Epithelial Cells. *AM.J.Respir.Cell.Mol.Biol.* 10, 38-47.

Crapo, JD., Barry, BE., Gehr, P., Bachofen, M., and Weibel, ER. (1982). Cell Number and Cell Characteristics of the Normal Human Lung. *Am Rev Respir Dis* 126, 332-337.

Crapo, JD., Young, SL., Fram, EK., Pinkerton, KE., Barry, BE., and Crapo, RO. (1983). Morphometric Characteristics of Cells in the Alveolar Region of Mammalian Lungs. *Am Rev Respir Dis* 128, S42-S46

Cross, CE., van der Vliet, A., O'Neill, C., Louie, S., and Halliwell, B. (1994). Oxidants, Antioxidants, and Respiratory Tract Lining Fluids. *Environmental Health Perspective.* 102, 185-191.

Crow, JP. and Beckman, JS. (1995). Reactions between Nitric Oxide, Superoxide, and Peroxynitrite: Footprints of Peroxynitrite *in Vivo*. In Nitric Oxide: Biochemistry, Molecular Biology, and Therapeutic Implications. L. Ignarro and F. Murad, eds. (London: Academic Press), pp. 17-44.

Crystal, RG. (1991). Oxidants and Respiratory Tract Epithelial Injury: Pathogenesis and Strategies for Therapeutic Intervention. *Am.J.Med.* 91, 3C-39S-3C-53S.

Culotta, E. and Koshland, DE. (1992). NO News is Good News. *Science* 258, 1862-1865.

Dantzker, DR. (1990). Oxygen Transport and Utilization in ARDS. *Eur Respir J* 3, 485s-489s.

Davis, WB. and Pacht, ER. (1998). Extracellular Antioxidant Defenses. book *Chapter 2.3*, 61-68.

De Vera, ME., Shapiro, RA., Nussler, AK., Mudgett, JS., Simmons, RL., Morris, SM., Billiar, TR., and Geller, DA. (1996). Transcriptional Regulation of Human Inducible Nitric Oxide Synthase (NOS2) Gene by Cytokines: Initial Analysis of the Human NOS2 Promoter. *Proc.Natl.Acad.Sci.USA* 93, 1054-1059.

del Pozo, V., de Arrunda-Chaves, E., de Andres, B., Cardabe, B., Lopez-Farre, A., GallardoS.o, I., Viadarte, L., Jurado, A., Sastre, J., Palomino, P., and Lahoz, C. (1997). Eosinophils Transcribe and Translate Messenger RNA for Inducible Nitric Oxide Synthase. *J.Immunol.* 158, 859-864.

Deneke, SM. and Fanburg, BL. (1989). Regulation of Cellular Glutathione. *Am J Physiol* 257, L163-L173

Deneke, SM., Susanto, I., Vogel, K., and Williams, C. (1995). Mechanism of Use of Extracellular Glutathione by Lung Epithelial Cells and Pulmonary Artery Endothelial Cells. *AM.J.Respir.Cell.Mol.Biol.* 12, 662-668.

Dinsdale, D., Green, JA., Manson, MM., and Lee, MJ. (1992). The Ultrastructural Immunolocalisation of g-glutamyltranspeptidase in rat Lung: Correlation with the Histochemical demonstration of Enzyme Activity. *Histochemical Journal.* 24, 144-152.

Droge, W., Schulze-Osthoff, K., Mihm, S., Galter, D., Schenk, H., Eck, HP., Roth, S., and Gmunder, H. (1994a). Functions of Glutathione and Glutathione disulfide in Immunology and Immunopathology. *FASEB* 8, 1131-1138.

Dunhill, MS. (1960). The Pathology of Asthma with Special Reference to Changes in the Bronchial Mucosa. *J.Cell.Physiol.* 13, 224-225.

Duval, DL., Sieg, DJ., and Billings, RE. (1995). Regulation of Hepatic Nitric Oxide Synthase by Reactive Oxygen Intermediates and Glutathione. *Arch.Biochem.Biophys.* 316, 699-706.

Farber, JL., Kyle, ME., and Coleman, JB. (1990). Biology of Disease - Mechanisms of Cell Injury by Activated Oxygen Species. *Laboratory Investigations* 62, 670-678.

Felley-Bosco, E., Ambs, S., Lowenstein, CJ., Keefer, LK., and Harris, CC. (1994). Constitutive Wxpression of Inducible Nitric Oxide Synthase in Human Bronchial Epithelial Cells Induces c-fos and Stimulates the cGMP Pathway. *AM.J.Respir.Cell.Mol.Biol.* 11, 159-164.

Fidelus, R. (1998). The Generation of Oxygen Radicals: A Positive Signal for Lymphocyte Activation. *Cell Immunology.* 113, 178-182.

Flenley, DC., Downign, I., and Greening, AP. (1986). The Pathogenesis of Emphysema. *Eur.Pysiopathol.Respir.* 22, 245-252.

Flodstrom, M., Welsh, N., and Eizirik, DL. (1996). Cytokines Activate the Nuclear Factor κ B (NF- κ B) and Induce Nitric Oxide Production in Human Pancreatic Islets. *Febs Letters.* 385, 4-6.

Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M., and Packer, L. (1997). Redox Regulation of NF- κ B Activation. *Free Rad Biol Med* 22, 1115-1126.

Fujihara, M., Connolly, N., Ito, N., and Suzuki, T. (1994). Properties of Protein Kinase C Isoforms (β II, ϵ ,and ζ) in a Macrophage Cell Line (J774) and Their Roles in LPS-Induced Nitric Oxide Production. *J.Immunol.* 152, 1898-1906.

Furchgott, RF. and Zawadzki, JV. (1980). The Obligatory Role of Endothelial Cells in the Relaxation of Arterial Smooth Muscle by Acetylcholine. *nature* 288, 373-376.

Galter, D., Mihm, S., and Droge, W. (1997). Distinct Effects of Glutathione Disulphide on the Nuclear Transcription Factors κ B and the Activator Protein-1. *European Journal of Biochemistry*.

Gaston, B., Drazen, JM., Loscalzo, J., and Stamler, JS. (1994). The Biology of Nitrogen Oxides in the Airways. *Am.J.Resp.Crit.Care.Med* 149(2 pt 1), 538-551.

Gaston, B., Reilly, J., Drazen, JM., Fackler, J., Ramdev, P., Arnette, D., Mullins, ME., Sugarbaker, DJ., Chee, C., Singel, DJ., Loscalzo, J., and Stamler, JS. (1993). Endogenous Nitrogen Oxides and Bronchodilator S-Nitrosothiols in Human Airways. *Proc.Natl.Acad.Sci.USA* 90, 10957-10961.

Geller, DA., Lowenstein, CJ., Shapiro, RA., Nussler, AK., Di Silvio, M., Wang, SC., Nakayama, DK., Simmons, RL., Snyder, SH., and Billiar, TR. (1993). Molecular Cloning and Expression of Inducible Nitric Oxide Synthase from Human Hepatocytes. *Proc.Natl.Acad.Sci.USA* 90, 3491-3495.

Gille, J. and Joenje, H. (1992). Cell Culture Models for Oxidative Stress: Superoxide and Hydrogen Peroxide Versus Normobaric Hyperoxia. *Mutation Research*. 275, 405-414.

Gillissen, A., Jaworska, M., Orth, M., Coffiner, M., Maes, P., App, EM., Cantin, AM., and Schultze-Werninghaus, G. (1997). Nacystelyn, a Novel Lysine Salt of N-acetylcysteine, to Augment Cellular Antioxidant Defense *In Vitro*. *Respiratory Medicine* 91, 159-168.

Ginn-Pease, ME. and Whisler, RL. (1996). Optimal NF κ B Mediated Transcriptional Responses in Jurkat T Cells exposed to Oxidative Stress are Dependent on Intracellular Glutathione and Costimulatory Signals. *Biochem.Biophys.Res.Comm.* 226, 695-702.

Granger, DN. and Kubes, P. (1996). Nitric Oxide as Antiinflammatory Agent. *Meth.Enzymol.* 269, 434-442.

Green, LC., Wagner, DA., Glogowski, J., Skipper, P.JS., and Tannenbaum, SR. (1982). Analysis of Nitrate, Nitrite, and [¹⁵N]Nitrate in Biological Fluids. *Analytical Biochemistry.* 126, 131-138.

Griffith, OW. (1980). Determination of Glutathione and Glutathione Disulfide Using Glutathione reductase and 2-Vinylpyridine. *Anal.Biochem.* 106, 207-212.

Gryglewski, RJ., Palmer, R., and Moncada, S. (1986). Superoxide Anion is Involved in the Breakdown of Endothelium-derived Vascular Relaxing Factor. *nature* 320, 454-456.

Guo, FH., De Raeve, HR., Rice, TW., Stuehr, DJ., Thunnissen, F., and Erzurum, SC. (1995). Continuous Nitric Oxide Synthesis by Inducible Nitric Oxide Synthase in Normal Human Airway Epithelium *In Vivo*. *Proc.Natl.Acad.Sci.USA* 92, 7809-7813.

Gutteridge, J. (1993). Invited Review Free Radicals in Disease Processes: A Compilation of Cause and Consequence. *Free.Rad.Res.Comms.* 19, 141-158.

Gutteridge, J. (1994). Biological Origin of Free Radicals, and Mechanisms of Antioxidant Protection. *Chemico-biological Interactions.* 91, 133-140.

Hagen, TM., Aw, TY., and Jones, DP. (1988). Glutathione Uptake and Protection against Oxidative Injury in Isolated Kidney Cells. *Kidney Int.* 34, 74-81.

Hagen, TM. and Jones, DP. (1987). Transepithelila Transport of Glutathione in Vascularly Perfused Small Intestine of Rat. *Am J Physiol* 252, G607-G613

Halliwell, B. (1991a). Reactive Oxygen Species in Living Systems; Source, Biochemistry, and Role in Human Disease. *Am.J.Med.* 91, 3C-14S-3C-22S.

Halliwell, B. and Arouma, O. (1991b). DNA Damage by Oxygen-derived Species. *Febs Letters.* 281, 9-19.

Halliwell, B., Guutteridge, J., and Cross, CE. (1992). Free Radicals, Antioxidants, and Human Disease: Where are we now? *J.Lab Clin Med* 119, 598-620.

Hamid, Q., Springhall, DR., Riveros-Moreno, V., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S., and Polak, JM. (1993). Induction of Nitric Oxide Synthase in Asthma. *The Lancet* 342, 1510-1513.

Harris, JW. and Teng, SS. (1997). Sulfhydryl Groups During the S Phase: Comparison of Cells from G₁, Plateau-Phase G₁ and G₀.

Hayashi, T., Ueno, Y., and Okamoto, T. (1993). Oxidoreductive Regulation of Nuclear Factor k B. *J.Biol.Chem.* 268, 11380-11388.

Hecker, M., Preiss, C., Klemm, P., and Busse, R. (1996a). Inhibition by Antioxidants of Nitric Oxide Synthase Expression in Murine Macrophages: Role of Nuclear Factor kB and Interferon Regulatory Factor 1. *Br.J.Pharmacol.* 118, 2178-2184.

Hecker, M., Preiss, C., Schini-kerth, VB., and Busse, R. (1996b). Antioxidants Differentially affect Nuclear Factor kB-mediated Nitric Oxide Synthase Expression in Vasuclar Smooth Muscle Cells. *Febs Letters.* 380, 224-228.

Heffner, JE. and Repine, JE. (1989). Pulmonary Strategies of Antioxidant Defense. *Am Rev Respir Dis* 140, 531-554.

Hibbs, JB., Taintor, RR., and Vavrin, Z. (1987). Macrophage Cytotoxicity: Role for L-arginine Deiminase and imino Nitrogen Oxidation to Nitrite. *Science* 235, 473-476.

Hodge-Jahngen, J., Obin, MS., Gong, X., Shang, F., Nowell, TR., Gong, J., Abasi, H.J., and Taylor, A. (1997). Regulation of Ubiquitin-conjugating Enzymes by Glutathione Following Oxidative Stress. *J.Biol.Chem.* 272, 28218-28226.

Hogg, N., Singh, RJ., and Kalyanaraman, B. (1996). The Role of Glutathione in the Transport and Catabolism of Nitric Oxide. *Febs Letters.* 382, 223-228.

Holgate, ST. (1993). Asthma: Past, Present and Future. *Eur Respir J* 6, 1507-1520.

Horton, JK., Meredith, MJ., and Bend, JR. (1987). Glutathione Biosynthesis from Sulfur-Containing Amino Acids in Enriched Populations of Clara and Type II Cells and Macrophages Freshly Isolated from Rabbit Lung. *J.Parmacol.Exp.Ther.* 240, 376-380.

Hothersall, JS., Cunha, FQ., Neild, GH., and Norohna-Dutra, AA. (1997). Induction of Nitric Oxide Synthesis in J774 Cells Lowers Intracellular Glutathione: Effect of modulated Glutathione Redox Status on Nitric Oxide Synthase Induction. *Biochem.J.* 322, 477-481.

Ignarro, L., Lipton, H., Edwards, JC., Baricos, WH., Hyman, AL., Kadowitz, PJ., and Gruetter, CA. (1981). Mechanism of Vascular Smooth Muscle Relaxation by Organic Nitrates, Nitrites, Nitroprusside and Nitric Oxide: Evidence for the Involvement of S-Nitrosothiols as Active Intermediates. *J.Parmacol.Exp.Ther.* 218, 739-749.

Ignarro, L. and Murad, F. (1995). Nitric Oxide - Biochemistry, Molecular Biology and Therapeutic Implications. *Advances in Pharmacology*

Ignarro, LJ., Buga, GM., Wood, KS., Byrns, RE., and Chaudhuri, G. (1987). Endothelium-derived Relaxing Factor Produced and Released From Artery and Vein is Nitric Oxide. *Proc.Natl.Acad.Sci.USA* 84, 9265-9269.

Iyengar, R., Steuhr, DJ., and Marletta, MA. (1987). Macrophage Synthesis of Nitrite, Nitrate, and N-nitrosamines: Precursors and Role of the Respiratory Burst. *Proc.Natl.Acad.Sci.USA* 84, 6369-6373.

Jany, B., Betz, R., and Schreck, R. (1995). Activation of the Transcription Factor NF- κ B in Human Tracheobronchial Epithelial Cells by Inflammatory Stimuli. *Eur Respir J* 8, 387-391.

Jeffrey, PK. (1992). Chronic Obstructive Pulmonary Disease and Cigarette Smoke-induced Epithelial Damage. *Eur.Respir.Rev.* 2, 136-143.

Jeffrey, PK. (1996). Bronchial Biopsies and Airway Inflammation. *Eur Respir J* 9, 1583-1587.

Jeffrey, PK. (1998). Microscopic Structure of Normal Lung. In Brewis, RAL., Gibson, GJ. *Respiratory Medicine* 2nd edition p54-72. Saunders LTD, London.

Jeffrey, PK., Wardlaw, J., Nelson, F.JV., and Kay, AB. (1989). Brocnchial Biopsies in Asthma. *Am Rev Respir Dis* 140, 1745-1753.

Johannigman, JA., Davis, K., Campbell, RS., Luchette, F., Hurst, JM., and Branson, RD. (1997). Inhaled Nitric Oxide in Acute Respiratory Distress Syndrome. *J.Trauma.* 43, 904-910.

Kang, Y.J. (1993). Buthionine Sulfoximine Spares Intracellular Glutamate: A Possible Mechanism for Cell Growth Stimulation. *Cell.molec.Biol.Res.* 39, 675-684.

- Kang, Y.J. (1994). Exogenous Glutathione attenuates the Antiproliferative effect of BSO. *Toxicology* 88, 177-189.
- Kang, Y.J. and Enger , M.D. (1990). Glutathione Content and Growth in A549 Human Lung Carcinoma Cells. *Exp.Cell Res.* 187, 177-179.
- Kang, YJ. and Enger, MD. (1992). Buthionine Sulfoximine-Induced Cytostasis Does Not Correlate with Glutathione Depletion. *Am J Physiol* 262, C122-C127
- Kazzaz, JA., Xu, J., Palaia, TA., Mantell, L., Fein, AM., and Horowitz, S. (1996). Cellualr Oxygen Toxicity - Oxidant Injury without Apoptosis. *J.Biol.Chem.* 271, 15182-15186.
- Kharitonov, SA., Evans, D., O'Connor, BJ., and Barnes, P.J. (1996). Increased Exhaled Nitric Oxide in Asthma is Mainly Derived from the Lower Respiratory Tract. *Am.J.Resp.Crit.Care.Med* 153, 177-180.
- Kinnula, VL., Chang, L., Everitt, JI., and Crapo, JD. (1992). Oxidants and Antioxidants in Alveolar Epithelial Type II Cells: In Situ, Freshly Isolated, and Cultured Cells. *Am J Physiol* 262, L69-L77
- Kinnula, VL., Crapo, JD., and Raivio, KO. (1995). Biology of Disease: Generation and Disposal of Reactive Oxygen Metabolites in the Lung. *Lab.Invest.* 73, 3-19.
- Kinnula, VL., Everitt, JI., Whorton, AR., and Crapo, JD. (1991). Hydrogen Peroxide Production by Alveolar Type II Cells, Alveolar Macrophages, and Endothelial Cells. *Am J Physiol* 261, L84-L91
- Kooy, NW., Royall.JA., Ye, YZ., Kelly, DR., and Beckman, JS. (1995). Evidence for *In Vivo* Peroxynitirte Production in Human Acute Lung Injury. *Am.J.Resp.Crit.Care.Med* 151, 1250-1254.

Kretz-Remy, C., Mehlen, P., Mirault, M., and Arrigo, A. (1996). Inhibition of I κ B- α Phosphorylation and Degradation and Subsequent NF- κ B Activation by Glutathione Peroxidase Over Expression. *J.Biol.Chem.* 133, 1083-1093.

Kumuda, CD., Lewis-Molock, Y., and White, CW. (1995). Activation of NF κ B and Elevation of MnSOD Gene Expression by Thiol Reducing Agents in Lung Adenocarcinoma (A549) Cells. *Am J Physiol* 269, L588-L602

Kuo, PC. and Abe, KY. (1996a). Nitric Oxide-associated Regulation of Hepatocyte Glutathione Synthesis is a Guanylyl Cyclase-independent Event. *Surgery* 120, 309-314.

Kuo, PC., Abe, KY., and Schroeder, RA. (1996b). Interleukin-1-induced Nitric Oxide Production Modulates Glutathione Synthesis in Cultured Rat Hepatocytes. *Am J Physiol* 371, C851-C862

Li, F., Rosenberg, E., Smith, C., Notarfrancesco, K., Reisher, SR., Shuman, H., and Feinstein, SL. (1995). Correlation of Expression of Transcription Factor C/EBP Alpha and Surfactant Protein Genes in Lung Cells. *Am J Physiol* 269, L241-L247

Li, XY., Donaldson, K., Brown, D., and MacNee, W. (1995). The Role of Tumor Necrosis Factor in Increased Airspace Epithelial Permeability in Acute Lung Inflammation. *AM.J.Respir.Cell.Mol.Biol.* 13, 185-195.

Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W., and Todaro, G. (1976). A Continuous Tumor-Cell Line from a Human Lung Carcinoma with Properties of Type II Alveolar Epithelial Cells. *Int.J.Cancer.* 17, 62-70.

Lincoln, TM., Cornwell, TL., Komalavilas, P., and Boerth, N. (1996). Cyclic GMP-Dependent Protein Kinase in Nitric Oxide Signaling. *Meth.Enzymol.* 129, 149-166.

Ling, CC., Wong, R., and Basa, RD. (1990). Glutathione Depletion and Cytotoxicity of Buthionine Sulphoximine and SR2508 in Rodent and Human Cells. *I.J.Radiation Oncology Biology Physics* 18, 325-330.

Liu, RM., Hu, H., Robinson, TW., and Foramn, HJ. (1996). Increased g-Glutamylcysteine Synthetase and g-Glutamyl Transpeptidase Activites Enhance Resistance of Rat Lung Epithelial L2 Cells to Quinone Toxicity. *AM.J.Respir.Cell.Mol.Biol.* 14, 192-197.

Liu, SF., Adcock, IM., Old, RW., Barnes, PJ., and Evans, TW. (1996). Differential Regulation of the Constitutive and Inducible Nitric Oxide Synthase mRNA by Lipopoyasacchrde Treatment *in vivo* in the Rat. *Critical Care Medicine* 24, 1219-1225.

Lunberg, JO., Nordvall, SL., Weitzberg, E., Kollberg, HA., and Alving, K. (1996). Exhaled Nitric Oxide in Paediatric Asthma and Cystic Fibrosis. *Arch.Dis.Childhood.* 75, 323-326.

Luperchio, S., Tamir, S., and Tannenbaum, SR. (1996). NO-induced Oxidative Stress and Glutathione Metabolism in Rodent and Human Cells. *Free Rad Biol Med* 21, 513-519.

Lyons, CR., Orloff, GJ., and Cunningham, JM. (1992). Molecular Cloning and Functional Expression of an Inducible Nitric Oxide Synthase from a Murine Macrophage Cell Line. *J.Biol.Chem.* 267, 6370-6374.

MacKichie, ML., Logeat, F., and Israel, A. (1996). Phophorylation of p105 PEST Sequences via a Redox-insensitive Pathway Up-regulates Processing to p50 NF- κ B. *J.Biol.Chem.* 271, 6084-6091.

Maier, R., Bilbe, G., Rediske, J., and Lotz, M. (1994). Inducible Nitric Oxide Synthase from Human Articular Chondrocytes cDNA Cloning and Analysis of mRNA Expression. *Biochem.Biophys.Acta* 1208, 145-150.

Marletta, MA. (1989). Nitric Oxide: Biosynthesis and Biological Significance. *TIBS* 14, 488-492.

Marletta, MA. (1993). Nitric Oxide Synthase Structure and Mechanism. *J.Biol.Chem.* 268, 12231-12234.

Marletta, MA., Yoon, PS., Iyengar, R., Leaf, CD., and Wishnok, JS. (1997). Macrophage Oxidation of L-Arginine to Nitrite and Nitrate: Nitric Oxide is an Intermediate. *Biochemistry* 27, 8706-8711.

Massaro, AF., Gaston, B., Kita, D., Fanta, C., Stamler, JS., and Drazen, JM. (1995). Expired Nitric Oxide Levels During treatment of Acute Asthma. *Am.J.Resp.Crit.Care.Med* 152(2), 800-803.

Matalon, S., Holm, BA., Baker, RR., Whitfield, MK., and Freeman, BA. (1990). Characterization of Antioxidant Activities of Pulmonary Surfactant Mixtures. *Biochem.Biophys.Acta* 1035, 121-127.

Matthews, JR., Botting, CH., Panico, M., Morris, HR., and Hay, RT. (1997). Inhibition of NF- κ B DNA Binding by Nitric Oxide. *Nucleic Acids Research* 24, 2236-2242.

Matthews, JR., Wakasugi, N., Virelizer, JL., Yodoi, J., and Hay, RT. (1992). Thioredoxin Regulates the DNA Binding Activity of NF- κ B by Reduction of a Disulphide Bond Involving Cysteine 62. *Nuc.Acids.Res.* 20, 3821-3830.

- Meister, A. and Anderson, M.E. (1983). Glutathione. *Ann.Rev.Biochem.* 52, 711-760.
- Mellits, KH., Hay, RT., and Goodbourn, S. (1993). Proteolytic Degradation of NAD3(IkB α) and Enhanced Processing of the NF- κ B Precursor p105 are Obligatory Steps in the Activation of NF- κ B. *Nucleic Acids Research* 21, 5059-5066.
- Meyer, DJ., Kramer, H., and Ketterer, B. (1994). Human Glutathione Transferase Catalysis of the Formation of S-nitrosglutathione from Organic Nitrites Plus Glutathione. *Febs Letters.* 351, 427-428.
- Meyer, M., Schreck, R., and Baeuerle, PA. (1993). H₂O₂ and Antioxidants have Opposite Effects on Activation of NF- κ B and AP-1 in Intact Cells: AP-1 as Secondary Antioxidant-responsive Factor. *EMBO J* 12, 2005-2015.
- Moldeus, P. and Jernstrom, B. (1983). Interaction of Glutathione with Reactive Intermediates. In *Glutathione*. Anonymous New York: Raven Press), pp. 99-107.
- Moncada, S., Palmer, R., and Higgs, EA. (1991). Nitric Oxide: Physiology, Pathophysiology, and Pharmacology. *Pharm.Reviews* 43, 109-142.
- Morgan, SI. and Darling, DC. (1996). Animal Cell Culture. In *Introduction to Biotechniques*. Anonymous
- Morris, PE. and Bernard, GR. (1994). Significance of Glutathione in Lung Disease and Implications for Therapy. *Am.J.Med.Sci.* 307, 119-127.
- Mulier, B., Rahman, I., Watchorn, T., Donaldson, K., MacNee, W., and Jeffrey, PK. (1998). Hydrogen Peroxide-induced epithelial Injury: the Protective Role of Intracellular Nonprotein Thiols (NPSH). *Eur Respir J* 11, 384-391.

Murphy, ME., Piper, HM., Watanabe, H., and Sies, H. (1991). Nitric Oxide Production by Cultured Aortic Endothelial Cells in Response to Thiol Depletion and Replenishment. *J.Biol.Chem.* 266, 19378-19383.

Nathan, C. and Xie, QW. (1994). Regulation of Biosynthesis of Nitric Oxide. *J.Biol.Chem.* 19, 13725-13728.

Natoli, G., Costanzo, A., Moretti, F., Fulco, M., Balsano, C., and Leviero, M. (1997). Tumour Necrosis Factor (TNF) Receptor 1 Signaling Downstream of TNF Receptor-associated Factor 2. Nuclear Factor kappaB- inducing Kinase Requirement for Activation of Activating Protein 1 and NFkappaB but not of c-jun N-terminal Kinase/stress-activated Protein Kinase. *J.Biol.Chem.* 272, 26079-26082.

Newton, R., Adcock, IM., and Barnes, PJ. (1997). Superinduction of NF- κ B by Antinomycin D and Cycloheximide in Epithelial Cells. ?

Novak, Z., Nemeth, I., Gyurkovits, K., Varga, SI., and Matkovics, B. (1991). Examination of the Role of Oxygen free radicals in Bronchial Asthma in Childhood. *Clinica Chemica Acta* 201, 247-252.

Nussler, AK. and Billiar, TR. (1993). Inflammation, Immunoregulation, and Inducible Nitric Oxide Synthase. *J.Leuk.Biol.* 54, 171-178.

Nussler, AK., Liu, ZZ., Di Silvio, M., Seetland, MA., Geeler, DA., Lancaster, JR., Billiar, TR., Freeswick, PD., Lowenstein, CL., and Simmons, RL. (1994). Hepatocyte Inducible Nitric Oxide Synthase is Influenced In Vitro by Cell Density. *Am J Physiol* 266, C394-C401

Ohara, Y., Sayegh, HS., Yamin, JJ., and Harrison, DG. (1995). Regulation of Endothelial Constitutive Nitric Oxide Synthase by Protein Kinase C. *Hypertension* 25, 415-420.

Pacht, ER., Timerman, AP., Lykens, MG., and Merola, AJ. (1991). Deficiency of Alveolar Fluid Glutathione in Patients with Sepsis and the Adult Respiratory Distress Syndrome. *Chest* 100, 1397-1403.

Pahl, HL. and Baeuerle, PA. (1994). Oxygen and the Control of Gene Expression. *Bioassays* 16, 497-502.

Palmer, RM., Ashton, DS., and Moncada, S. (1988). Vascular Endothelial Cells Synthesise Nitric Oxide from L-arginine. *nature* 333, 664-666.

Palmer, RM., Ferrige, AG., and Moncada, S. (1987). Nitric Oxide Release Accounts for the Biological Activity of Endothelium-derived Relaxing Factor. *nature* 327, 524-526.

Peng, HB., Libby, P., and Liao, JK. (1995). Induction and Stabilization of I κ B α by Nitric Oxide Mediates Inhibition of NF- κ B. *J.Biol.Chem.* 270, 14214-14219.

Persinger, R., Marsh, J., Shull, S., Janssen, Y., and Mossman, BT. (1996). Differences in Constitutive and Inducible Expression of Antioxidant Enzymes in Rodent Pleural, Mesothelial Cells, Tracheal Epithelial Cells and lung Fibroblasts after Exposure to Hydrogen Peroxide *In Vitro*. *Am.J.Resp.Crit.Care.Med* 1439 (abstract),

Phelps, DT., Deneke, SM., Daley, DL., and Fanburg, BL. (1992). Elevation of Glutathione Levels in Bovine Pulmonary Artery Endothelial Cells by N-Acetylcysteine. *AM.J.Respir.Cell.Mol.Biol.* 7, 293-299.

Phelps, DT., Ferro, TJ., Higgins, PJ., Shankar, R., Parker, DM., and Johnson, A. (1995). TNF- α Induces Peroxynitrite-mediated Depletion of Lung Endothelial Glutathione via Protein Kinase C. *Am J Physiol* 269, L551-L559

Pheng, L.H., Francoeur, C., and Denis, M. (1995). The Involvement of Nitric Oxide in a Mouse Model of Adult Respiratory Distress Syndrome. *Inflammation* 19, 599-610.

Pietarinen, P., Raivio, K.O., Devlin, R.B., Crapo, J.D., Chang, L., and Kinnula, V.L. (1995). Catalase and Glutathione Reductase Protection of Human Alveolar Macrophages during Oxidant Exposure *In Vitro*. *AM.J.Respir.Cell.Mol.Biol.* 13, 434-441.

Poot, M., Teubert, H., Rabinovitch, P.S., and Kavanagh, T.J. (1995). De Novo Synthesis of Glutathione is Required for Both Entry into and Progression through the Cell Cycle. *J.Cell.Physiol.* 163, 555-560.

Post, G.B., Keller, D.A., Connor, K.A., and Menzel, D.B. (1983). Effects of Culture Conditions on Glutathione Content in A549 Cells. *Biochem.Biophys.Res.Comm.* 114, 737-742.

Pryor, W.A., Terauchi, K.I., and Davis, W.H. (1976). Electron Spin Resonance (ESR) Study of Cigarette Smoke by use of Spin Tapping Technique. *Environ.Health.Perspect.* 16, 161-175.

Rahman, I. and MacNee, W. (1996). Role of Oxidants/Antioxidants in Smoking-Induced Lung Diseases. *Free Rad Biol Med* 21, 669-681.

Rahman, I. and MacNee, W. (1998). Role of Transcription Factors in Inflammatory Lung Diseases. *Thorax*

Regnier, C., Song, H., Gao, X., Goeddel, D., Cao, A., and Rothe, M. (1997). Identification and Characterisation of an I κ B Kinase. *Cell* 90, 373-383.

Riley, DJ. and Kerr, JS. (1998). Oxidant Injury in the Extracellular Matrix: Potential Role in the Pathogenesis of Pulmonary Emphysema. Springer-Verlag Lung, 163th ED 1-13. New York.

Robbins, RA., Barnes, PJ., Springhall, DR., Warren, JB., Kwon, OJ., Buttery, L., Wilson, AJ., Geller, DA., and Polak, JM. (1994). Expression of Inducible Nitric in Human Lung Epithelial Cells. *Biochem.Biophys.Res.Comm.* 203, 209-218.

Robbins, RA. and Grisham, MB. (1997). Nitric Oxide. *Int.J.Biochem.Cell.Biol* 29, 857-860.

Robbins, RA., Springhall, DR., Warren, JB., Buttery, L., Wilson, AJ., Adcock, IM., Riveros-Moreno, V., Moncada, S., Polak, J., and Barnes, PJ. (1994). Inducible Nitric Oxide Synthase is Increased in Murine Lung Epithelial Cells by Cytokine Stimulation. *Biochem.Biophys.Res.Comm.* 198 , 835-843.

Root, RK., Metcalf, JM., Oshine, N., and Chance, B. (1975). H₂O₂ Release from Human Granulocytes during Phagocytosis. *J.Clin.Invest.* 55, 945-955.

Royall,JA. and Kooy, NW. (1997). Peroxynitrite and Other Nitric Oxide-Derived Oxidants. In *Nitric Oxide and the Lung*. WM. Zapol and KD. Bloch, eds. (New york: Marcel Dekker), pp. 223-246.

Rubbo, H., Radi, R., Trujillo, M., Kayanaraman, B., Barnes, S., Kirk, M., and Freeman, BA. (1994). Nitric Oxide Regulation of Superoxide and Peroxynitire-dependent Lipid Peroxidation. *J.Biol.Chem.* 269, 26066-26075.

Ryrfeldt, A., Bannengerg, G., and Moldeus, P. (1993). Free Radicals and Lung Disease. *British Medical Bulletin.* 49, 588-603.

- Sachdev, V., Joshi, PC., Murray, T., and Thomae, KR. (1997). Expression of Inducible Nitric Oxide Synthase in Human Lungs. *J.Invest.Surgery*. 10, 315-318.
- Saleh, D., Barnes, P.J., and Giaid, A. (1997). Increased Production of the Potent Oxidant Peroxynitrite in the Lungs of Patients with Idiopathic Pulmonary Fibrosis. *Am.J.Resp.Crit.Care.Med* 155, 1763-1769.
- Salkowski, CA., Barber, SA., Deltore, GR., and Vogel, SN. (1996). Differential Dysregulation of Nitric Oxide Production in Macrophages and Targeted Disruptions in IFN Regulatory Factor-1 and -2 Genes. *J.Immunol*. 156, 3107-3110.
- Sato, M., Miyazaki, T., Nagaya, T., Murata, Y., Ida, N., Maeda, K., and Seo, H. (1996). Antioxidants Inhibit Tumor Necrosis Factor- α Mediated Stimulation of Interleukin-8, Monocyte Chemoattractant Protein-1, and Collagenase Expression in Cultured Human Synovial Cells. *J.Rheumatology*. 23, 432-438.
- Schenk, H., Klein, M., Erdbrugger, W., Droge, W., and Schulze-Osthoff, K. (1994). Distinct Effects of Thioredoxin and Antioxidants on the Activation of Transcription Factors NF- κ B and AP-1. *Proc.Natl.Acad.Sci.USA* 91, 1672-1676.
- Schmidt, H. and Walter, U. (1994). NO at Work. *Cell* 78, 919-925.
- Schraufstatter, IU., Hyslop, PA., Hinshaw, DB., Spragg, RG., Skalar, LA., and Cochrane, CG. (1986). Hydrogen Peroxide-induced Injury of Cells and its Prevention by Inhibitors of Poly(ADP-ribose) Polymerase. *Proc.Natl.Acad.Sci.USA* 83, 4908-4912.
- Schreck, R. and Baeuerle, PA. (1994). Assessing Oxygen Radical as Mediators in Activation of Inducible Eukaryotic Transcription factor NF- κ B. *Meth.Enzymol*. 234, 151-163.

Schreck, R., Rieber, P., and Baeuerle, PA. (1991). Reactive Oxygen Intermediates as Apparently Widely used Messengers in the Activation of the NF- κ B Transcription Factor HIV-1. *EMBO J* 10, 2247-2258.

Sedgwick, J., Geiger, KM., and Busse, R. (1990). Superoxide Generation by Hypodense Eosinophils from Patients with Asthma. *Am Rev Respir Dis* 142, 120-125.

Sen, CK. and Packer, L. (1996). Antioxidant and Redox Regulation of Gene Transcription. *FASEB* 710, 709-720.

Smith, LJ., Houston, M., and Anderson, J. (1993). Increased Levels of Glutathione in Bronchoalveolar Lavage Fluid from Patients with Asthma. *Am Rev Respir Dis* 147, 1461-1464.

Smith, PK., Krohn, RI., Hermanson, GT., Mallia, AK., Gartner, FH., Provenzano, MD., Fujimoto, EK., Geoke, NM., Olson, BJ., and Klenk, DC. (1985). Measurement of Protein Using Bicinchoninic Acid. *Analytical Biochemistry*. 150, 76-85.

Spitsin, SV., Farber, JL., Bertovich, M., Moehren, G., Koprowski, H., and Michaels, FH. (1997). Human- and Mouse-inducible Nitric Oxide Synthase Promoters Require Activation of Phosphatidylcholine-specific Phospholipase C and NF- κ B. *Mol.Med.* 3, 315-326.

Staal, F., Roederer, M., and Herzenberg, LA. (1990). Intracellular Thiols Regulate Activation of Nuclear Factor κ B and Transcription of Human Immunodeficiency Virus. *Proc.Natl.Acad.Sci.USA* 87, 9943-9947.

Stamler, JS. (1994). Redox Signaling: Nitrosylation and Related Target Interactions of Nitric Oxide. *Cell* 78, 931-936.

Stamler, JS. (1997). S-Nitrosothiols and the Bioregulatory Actions of Nitrogen Oxides Through Reactions with Thiol Groups. ?

Stamler, JS., Dingel, DJ., and Loscalzo, J. (1992). Biochemistry of Nitric Oxide and its Redox-Activated Forms. *Science* 258, 1898-1902.

Steuhr, DJ., Kwon, NS., and Nathan, CF. (1990). FAD and GSH Participate in Macrophage Synthesis of Nitric Oxide. *Biochem.Biophys.Res.Comm.* 168, 558-565.

Steuhr, DJ. and Marletta, MA. (1987). Synthesis of Nitrite and Nitrate in Murine Macrophage Cell Lines. *Cancer Research.* 47, 5590-5594.

Suzuki, YJ., Mizuno, M., and Packer, L. (1994). Signal Transduction for Nuclear Factor- κ B Activation. Proposed Location of Antioxidant-Inhibitable Step. *J.Immunol.* 153, 5008-5015.

Tate, SS. and Meister, A. (1981). γ -Glutamyl Transpeptidase: Catalytic, Structural and Functional Aspects. *Molec.Cell.Biochem.* 39, 357-368.

Taylor, BS., De Vera, ME., Ganster, RW., Wang, Q., Shapiro, RA., Morris, SM., Billiar, TR., and Geller, D. (1998). Multiple NF- κ B Enhancer Elements Regulate Cytokine Induction of the Human Inducible Nitric Oxide Synthase Gene. *J.Biol.Chem.* 273, 15148-15156.

Tetley, TD. (1993). Proteinase Imbalance: its Role in Lung Disease. *Thorax* 48, 560-565.

Tietze, F. (1969). Enzymic Method for Quantitative Determination of Nanogram Amounts of Total and Oxidized Glutathione: Application to Mammalian Blood and Other Tissues. *Anal.Biochem.* 27, 502-522.

Toledano, MB. and Leonard, WJ. (1991). Modulation of Transcription Factor NF- κ B Binding Activity by Oxidation-reduction *in vitro*. Proc.Natl.Acad.Sci.USA 88, 4328-4332.

Vandeputte, C., Guizon, I., Genestie-Denis, I., Vannier, B., and Lorenzon, G. (1994). A Microtiter Plate Assay for Total Glutathione and Glutathione Disulfide Contents in Cultured/Isolated Cells: Performance Study of a New Miniaturized Protocol. Cell.Biol.Tox. 10, 415-421.

Vanderbist, F., Maes, P., and Neve, J. (1996). In Vitro Comparative Assessment of the Antioxidant Activity of Nacystelyn against Three Reactive Oxygen Species. Drug research 46(II), 783-788.

Warner, RL., Paine III, R., Chrisensen, PJ., Marletta, MA., Richards, MK., Wilcoxon, SE., and Ward, PA. (1995). Lung Sources and Cytokine Requirements for In Vivo Expression of Inducible Nitric Oxide Synthase. AM.J.Respir.Cell.Mol.Biol. 12, 649-661.

Wefers, H. and Sies, H. (1989). The Protection by Ascorbate and Gluthathione Against Lipid Peroxidation is Dependent on Vitamin E. Febs Letters. 174, 353-357.

White, AC., Maloney, EK., Boustani, MR., Hassoun, PM., and Fanburg, BL. (1995). Nitric Oxide Increases Cellular Glutathione Levels in Rat Lung Fibroblasts. AM.J.Respir.Cell.Mol.Biol. 13, 442-448.

White, KA. and Marletta, MA. (1992). Nitric Oxide Synthase is a cytochrome P-450 Type Hemoprotein. Biochemistry 31, 6627-6631.

Winrow, V., Winyard, P., Morris, C., and Blake, D. (1993). Free Radicals in Inflammation: Second Messengers and Mediators of Tissue Destruction. British Medical Bulletin. 49, 506-522.

Wolff, SP., Garner, A., and Dean, RT. (1986). Free Radicals, Lipids, Protein Degradation. *TIBS 11*, 27-31.

Wong, HR., Finder, JD., Wasserloos, K., Lowenstein, CJ., Geller, DA., Billiar, TR., Pitt, BR., and Davies, P. (1996). Transcriptional regulation of iNOS by IL-1 β in Cultured Rat Pulmonary Artery Smooth Muscle Cells. *Am J Physiol* 271, L166-L171

Xie, QW., Kashiwabara, Y., and Nathan, C. (1994). Role of Transcription factor NF- κ B/Rel in Induction of Nitric Oxide Synthase. *J.Biol.Chem.* 269, 4705-4708.

Yates, D., Kharitonov, SA., Thomas, PS., and Barnes, P.J. (1996). Endogenous Nitric Oxide is Decreased in Asthmatic Patients by an Inhibitor of Inducible Nitric Oxide Synthase. *Am.J.Resp.Crit.Care.Med* 154, 247-250.

Yoshida, M., Taguchi, O., Gabazza, EC., Yasui, H., Kobayashi, T., Kobayashi, H., Maruyama, K., and Adachi, Y. (1997). The Effect of Low-dose Inhalation of Nitric Oxide in Patients with Pulmonary Fibrosis. *Eur Respir J* 10, 2051-2054.

APPENDIX ONE

Compound/Material	Supplier
A549 cell line	American Type Culture Collection (ATCC)
16HBE140- cell line	Dr Greunert
Acrylamide/Bis solution	Gibco.BRL
Ammonium persulphate (APS)	Sigma
Aqua perm	Life Science International
[γ - ³² P]ATP	Amersham
Avidin Biotinylated Complex (ABC)	DAKO
Boric acid,	Sigma
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Sigma
Buthionine sulfoximine (BSO)	Sigma
Calcium/magnesium free Phosphate Buffered Saline (PBS)	Sigma
Chloroform	Sigma
Diaminobenzedine (DAB)	Sigma
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
Dpx (a synthetic mountant)	Merck.BDH
5, 5'-Dithiobis (2-nitrobenzoic acid) (DTNB)	Sigma
Deoxy (DNTPs)	Gibco
Dulbecco's modified eagles medium (DMEM)	Sigma
Ethanol	Sigma
Ethidium bromide	Sigma
Ethylene diamine tetracetic acid (EDTA)	Sigma
5x First strand buffer	Gibco
GAPDH primers	MWG Biotech

Glutathione (GSH)	Sigma
Glutathione monoethylester (GSHMEE)	Sigma
Glutathione reductase (GR)	Promega
Glycerol	Sigma
HeLa nuclear protein extract	Sigma
HEPES buffer pH 7.0	Sigma
Hydrochloric acid (HCl)	Haymen LTD
IFN- γ	R & D systems
IL1- β	R & D systems
iNOS primers	MWG Biotech
Hydrogen peroxide (H ₂ O ₂)	Gibco
Isopropanol	Sigma
L-glutamine (L-G)	Sigma
Leupeptin	DAKO
L-NMMA	Sigma
LPS	Sigma
Magnesium chloride (MgCl ₂)	Calbiochem
Minimal essential medium (MEM)	Sigma
Mineral oil	Sigma
MMLV-reverse transcriptase	Gibco
Monoclonal Mouse anti-human cytokeratin antibody	Sigma
N-acetylcysteine (NAC)	Sigma
NADPH	Sigma
N-acetylcysteine L-lysinate	SMB Pharmaceuticals
N-(1-naphthyl)-ethanediamine	Sigma
NF- κ B oligonucleotide sequence	Promega
Noidet	Sigma
Non-essential amino acid mixture (AA)	Gibco
Nuclear fast red	Merck/BDH

Oligo dT	Gibco
Paraformaldehyde	Sigma
Penicillin (P)	Gibco
Phenyl methyl sulfonyl fluoride (PMSF)	Sigma
Phosphate Buffer (PB); Potassium dihydrogen orthophosphate, di-potassium orthophosphate 3-hydrate.	Merck/BDH
Phosphate buffered saline (PBS)	Sigma
Phosphoric acid	Sigma
10x T4 polynucleotide Kinase buffer	New England Biolabs
Potassium chloride (KCl)	Sigma
Protein assay kit	Pierce and Warriner
Pyrogallol	Sigma
Rabbit anti-mouse (RAM) antibody	Dako
Rabbit polyclonal NF- κ B antibody	Santa Cruz/Insigh biotech.
RNase inhibitor	Gibco
Sheared salmon sperm DNA	Immunotech
Sodium (Na) orthovanadate	Sigma
Sodium chloride	Sigma
Sodium fluoride (NaF)	Sigma
Sodium hydroxide	Sigma
Sodium pyruvate	Sigma
Sodium nitrite	Sigma
Sulfanilomide	Gibco
5-sulfosalicylic acid (SSA)	Sigma
Streptomycin (S)	Sigma
T4 polynucleotide Kinase	New England Biolabs
Taq polymerase	Gibco
Tetramethylethylenediamine (TEMED)	Sigma
Thiobarbituric acid (TBA)	Sigma

Trichloroacetic acid (TCA)	Sigma
Trietanolamie (TEA)	Sigma
Tris.HCL buffer pH= 8.0	Sigma
Triton-X-100	Sigma
TRIZOL	Gibco
Trypan blue	Sigma
Trypsin/EDTA	Gibco
Tumour necrosis factor- α	R and D systems

Supplier	Address
American Type Culture Collection (ATCC)	12301 Parklawn drive, Rockville, Maryland, 20852, USA 301-881-260
Amersham	Northern Europe region, Lincoln place, Green end, Aylesbury, Bucks. (0800) 515313
Calbiochem	Nova biochem (UK)Ltd, Boulevard Industrial park, Podge road, Beeston, Nottingham, NG9 2JR (01159) 43840
Dako	16 Manor Courtyard, Hughenden avenue, High Wycombe, Buckinghamshire, HP13 5RE (01494) 452016
Gibco/BRL	3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF (0800) 269210
Haymen LTD	70 Earhamp park, Witham, Essex, CM8 37E (01376) 517517
Immunotech/Coulter Electronics	Northwell Drive, Luton, Beds., LU3 3RH (01582) 567000
Life Science International	Unit 5, Ringway Center, Edison road, Basingstoke, Hampshire, RG21 6YH (01256) 817282
Merck/BDH	Hunter Boulevard, Magna park, Lutterworth, Leicestershire, LE17 4XN (0800) 223344
New England Biolabs	67 Knowl piece, Wilbury way, Hitchin, Herts., SG4 0TY (01462) 420616

Pierce and Warriner	44 Upper Northgate street, Chester, Chestershire, CH1 4EF (01244) 382525
Promega	Delat house, Enterprise house, Chilworth Reserach Center, Southampton, SO16 7NS (01703) 760225
Santa Cruz /Insight Biotech.	Wembley, Middlesex
Sigma/ Aldrich Co LTD	Fancy road, Poole, Dorset BH17 7NH (10202)733114
R and D systems	4-10 The Quadrant, Barton Lane, Abingdon, OX14 3YS. (01235) 551100